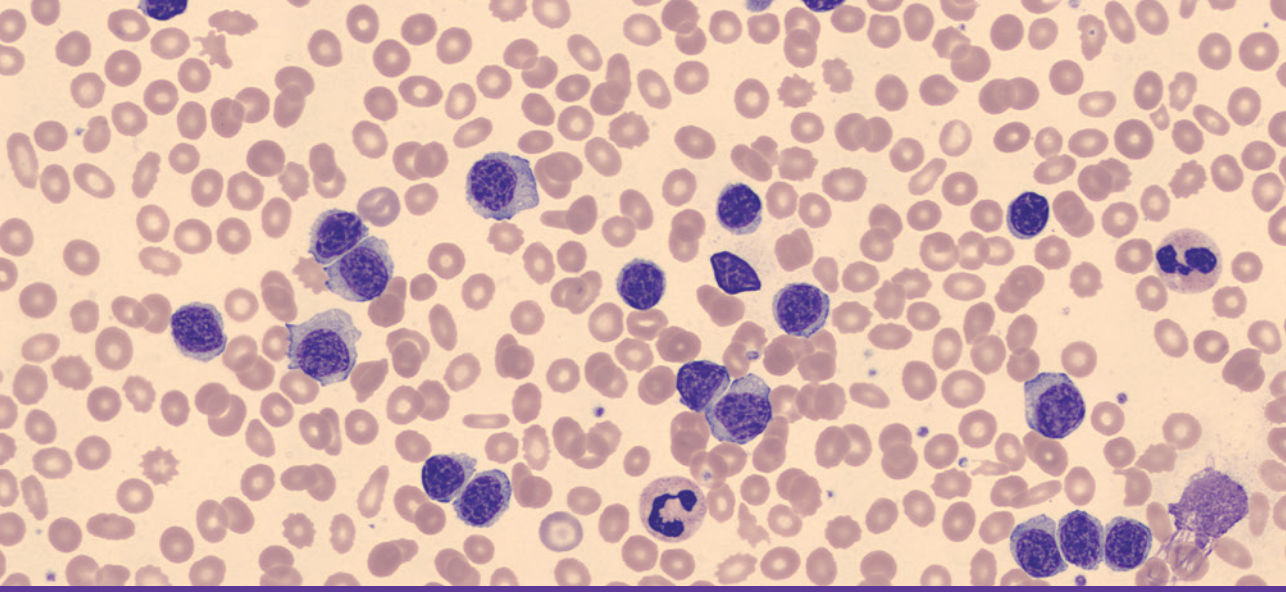
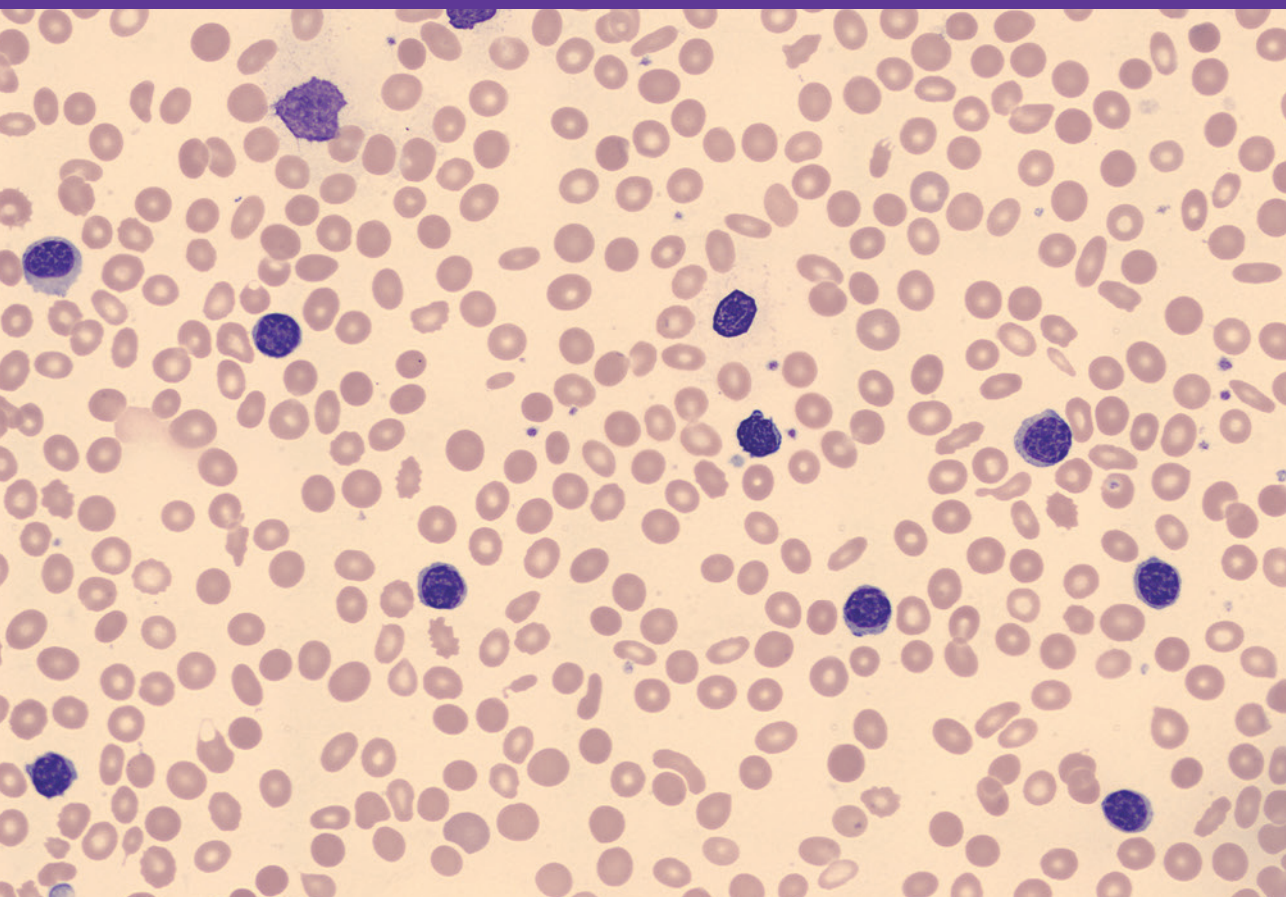


Current and future diagnostics of anaemia

Karlijn Stouten



Current And Future Diagnostics Of Anaemia

Karlijn Stouten

Colophon

The studies described in this thesis were performed at the Department of Internal Medicine and the Department of Clinical Chemistry of the Albert Schweitzer Hospital, Dordrecht, the Netherlands, under the supervision of Prof.Dr. P. Sonneveld of the Department of Haematology of the Erasmus University Medical Center, Rotterdam, the Netherlands.

This research was supported by the ORAS foundation, Result laboratory and the Leerhuis of the Albert Schweitzer Hospital.

Printing of this thesis was kindly supported by Amgen BV and Sysmex BV.

Cover photo: Cellavision
Printing: Ridderprint BV

Current And Future Diagnostics Of Anaemia

Huidige en toekomstige anemie diagnostiek

Proefschrift

ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam
op gezag van de
rector magnificus

Prof.dr. H.A.P. Pols

en volgens besluit van het College voor Promoties.

De openbare verdediging zal plaatsvinden op

Vrijdag 23 september 2016 om 09.30 uur

Karlijn Stouten

geboren te Deventer

Erasmus University Rotterdam

Erasmus

Promotiecommissie:

Promotor:	**Prof.Dr. P. Sonneveld**
Overige leden:	**Prof.Dr.Ir. H. Boersma**
	Prof.Dr. P.J.E. Bindels
	Prof.Dr. R. de Jonge
Copromotoren:	**Dr. M-D. Levin**
	Dr. J.A. Riedl

Contents

List of abbreviations

ACD	Anaemia of chronic disease
ACI	Anaemia of inflammation
CLL	Chronic lymphatic leukaemia
CRP	C-reactive protein
DCGP	Dutch college of general practitioners
DM	Digital microscope
EPO	Erythropoietin
GI	Gastrointestinal
GP	General practitioner
Hb	Haemoglobin
HR	Hazard ratio
IDA	Iron deficiency anaemia
IQR	Interquartile range
LDH	Lactate dehydrogenase
MCV	Mean corpuscular volume
MDS	Myelodysplastic syndrome
NPV	Negative predictive value
NRBC	Nucleated red blood cells
OR	Odds ratio
PBS	Peripheral blood smear
PPV	Positive predictive value
SD	Standard deviation
SMR	Standardised mortality ratio
WHO	World Health Organisation

1

General introduction

Anaemia is one of the most common health problems worldwide and is prevalent in every population at every age. It is characterised by a lowered concentration of haemoglobin (Hb), the oxygen-transporting protein carried by erythrocytes. The most common symptoms include fatigue, weakness, difficulty concentrating and general malaise. Globally the most common aetiology is iron deficiency anaemia (IDA). Other leading causes, such as anaemia of chronic disease (ACD) and chronic kidney disease, vary considerably by geography, age, and gender[1].

The World Health Organisation (WHO) has defined anaemia by determining haemoglobin cut-off values, shown in Table 1. The overall prevalence of WHO-defined anaemia in the complete world population was estimated to be 32.9% in 2010, with the highest burden found in children below the age of five years[1]. In a community-dwelling population, the prevalence was determined to be lowest in men in the age group 17-49 years. From the age of 50 years the prevalence starts rising for both men and women, peaking above 20% in the age group 85+ years[2] (Figure 1). Estimations of the incidence of anaemia vary widely and range from 12.8 to 90.2/1000 person-years in men and from 10.9 to 69.1/1000 person-years in women[3,4].

	Haemoglobin < g/dL
Age 5+ years	
Men	13.0
Women, non-pregnant	12.0
Women, pregnant	11.0
Age < 5 years	
Men	12.0
Women	12.0

***Table 1**, WHO-defined haemoglobin cut-off levels for the diagnosis of anaemia[1].*

Anaemia in general practice

The increasing prevalence of anaemia with age has long been explained as an innocent consequence of aging. During the last decade, however, many studies have been published detailing the detrimental effect on health aspects and mortality associated with anaemia (discussed below). The current remedy for anaemia is successful treatment of the underlying condition. Treatment of anaemia itself, i.e. the decreased haemoglobin level, is still limited[5,6]. Increased awareness among physicians about the prevalence of anaemia, its causes and its potential effects may improve this. Since anaemia is a common finding in general practice, it is crucial for general practitioners (GPs) to have a good understanding of the prevalence of the possible causes they may encounter. It is also important that the guidelines developed to aid GPs with the evaluation of anaemia are accurate. Many

consensus-based algorithms currently in use still focus on the traditional classification of anaemia aetiology based on the mean corpuscular volume (MCV) of the erythrocyte[7-10].

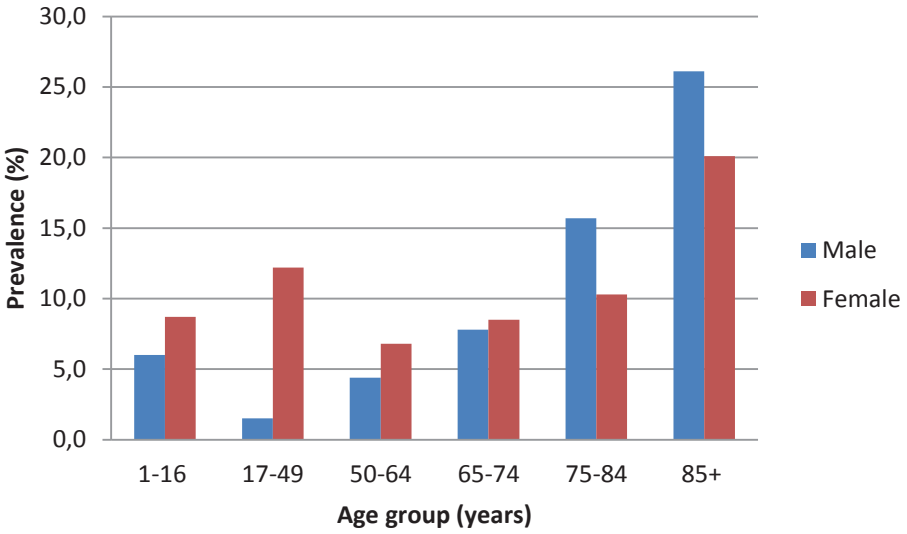


***Figure 1**, Percentage of persons considered anaemic according to the WHO standard, stratified by age and gender. Data taken from NHANES III (1998 to 2004) comprising a community-dwelling population in the United States. Adapted from Guralnik et al, 2004[2].*

This classification was developed by Wintrobe in the 1930s and has remained the prevailing division ever since[11]. While its exact validity in general practice is unclear, data from hospitalised patients indicates that MCV does not accurately reflect the underlying aetiology in the elderly and is practically useless when deciding on ordering further or more specific diagnostic tests[12-14].

Aetiology

Anaemia itself is considered a symptom and secondary to the underlying aetiology, which needs to be properly diagnosed to allow for appropriate treatment and correction of the haemoglobin level. It is often multifactorial and the underlying aetiology is very diverse. As a result, determining the correct diagnosis is often challenging. Most analyses of anaemic populations focus on four main categories of aetiology: anaemia of chronic disease (ACD, sometimes referred to as anaemia of inflammation (ACI)), renal anaemia, nutrient deficiency (iron, vitamin B12 and/or folic acid) and unknown anaemia. An overview of the prevalence rates of these causes in community-dwelling populations is shown in Figure 2[2-4,15-23].

Anaemia of chronic disease (ACD)

ACD is the most common cause in an elderly population (≥ 50 years), generally developing alongside conditions such as acute or chronic infections, organ failure, trauma, malignancy, and autoimmune or inflammatory disorders[24-33]. It is characterised by the retention of iron within macrophages and a reduced iron uptake from food. These effects are due to raised levels of hepcidin, a key regulator of iron metabolism[34], and limit the availability of soluble iron for erythrocyte production despite adequate iron stores, leading to a functional iron deficiency (Figure 3)[25].

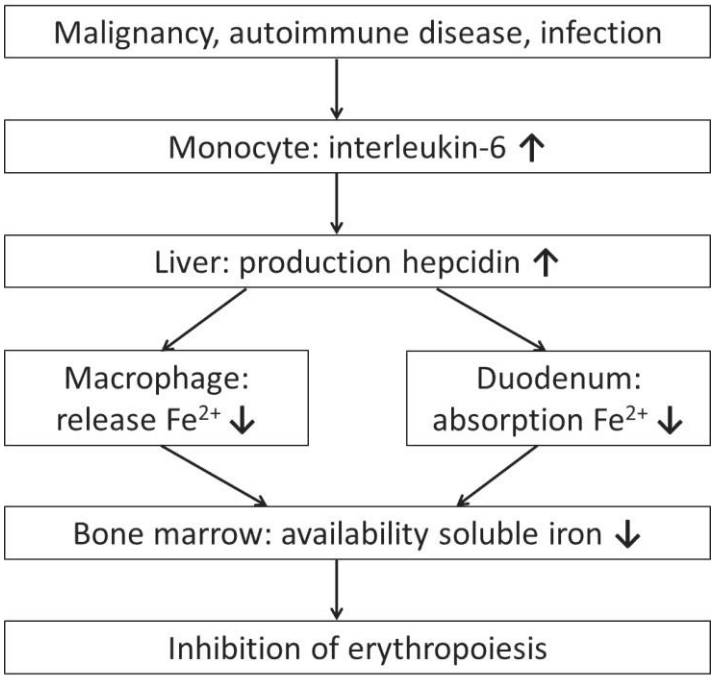
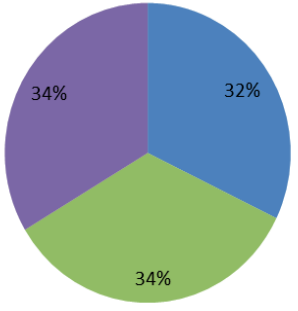


***Figure 3**, A schematic overview of the role of hepcidin in functional iron deficiency observed in anaemia in chronic disease*

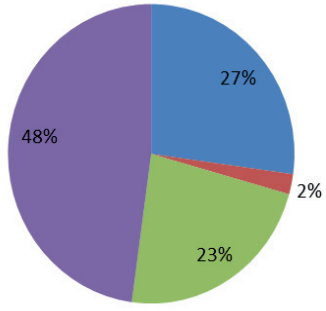
Renal anaemia

Renal anaemia is common among the elderly, due to a decline in renal function with age[35], and is usually related to a decreased erythropoietin (EPO) production[36-38]. Even at modest degrees of renal insufficiency, a decrease in haemoglobin can already be observed[36,39].

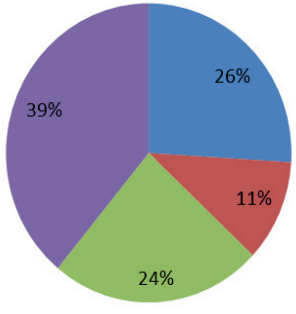
***Figure 2** (next page), an overview of the prevalence of ACD/ACI, renal anaemia, nutrient deficiency, unknown anaemia and other (if applicable) in several community-dwelling populations[2-4,15-23].*



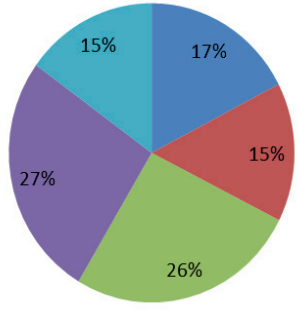
Population: non-institutionalised US population
Age: ≥ 65 years
N: 2096 patients, 274 anaemic
Reference: Guralnik *et al.* 2004[2]



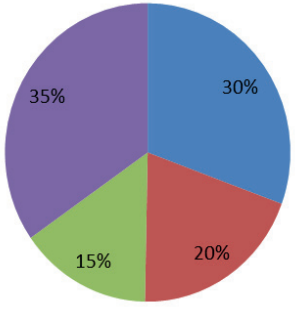
Population: general population Germany
Age: 45-75 years
N: 4814 patients, 152 anaemic
Reference: Eisele *et al.* 2013[3]



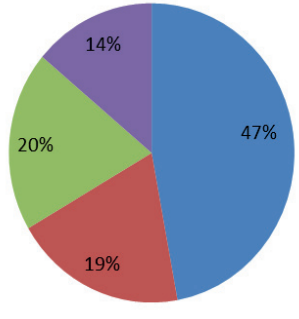
Population: non-institutionalised US population
Age: ≥ 50 years
N: 7171 patients, 862 anaemic
Reference: Shavelle *et al.* 2012[15]



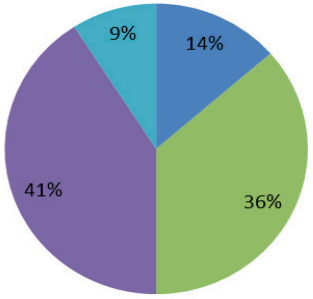
Population: general population Italy including nursing and residential homes
Age: ≥ 65 years
N: 8744 patients, 1243 anaemic
Reference: Tettamanti *et al.* 2010[17]



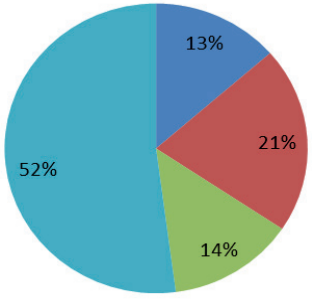
Population: moderately to severely disabled women living in community
Age: ≥ 65 years
N: 688 patients, 147 anaemic
Reference: Semba *et al.* 2007[16]



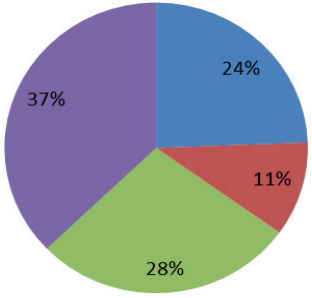
Population: general practice
Age: > 60 years
N: 530 patients, 72 anaemic
Reference: Kirkeby *et al.* 1991[18]



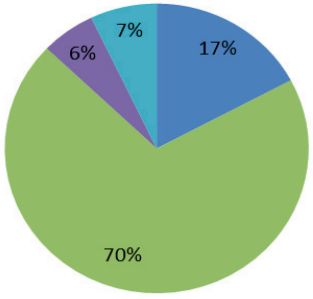
Population: home visit geriatric population
Age: ≥ 65 years median 85 years
N: 196 patients, 76 anaemic
Reference: Argento *et al.* 2008[19]



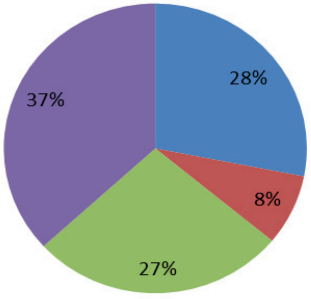
Population: general practice
Age: median 77 years
N: 854 patients, 51 anaemic
Reference: Merlo *et al.* 2008[22]



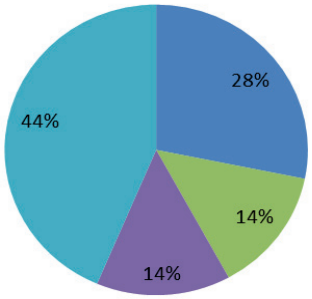
Population: population based study
Age: ≥ 65 years
N: 582 patients, 86 anaemic
Reference: Ferrucci *et al.* 2010[20]



Population: urban general practice
Age: no age limit
N: 4928 consultations, 106 anaemic
Reference: Kahn *et al.* 1990[23]



Population: population based study
Age: ≥ 65 years
N: 964 patients, 124 anaemic
Reference: Ferrucci *et al.* 2007[21]



Population: Population based study
Age: ≥ 65 years
N: 618 anaemic patients
Reference: Ania *et al.* 1997[4]

ACD/ACI Renal anaemia Nutrient deficiency Unknown anaemia Other

EPO replacement therapy may resolve anaemia secondary to renal insufficiency and is used in, for example, patients with end-stage renal disease[40,41]. Often patients with renal anaemia also display features of ACD, due to low grade inflammation associated with renal insufficiency[42].

Nutrient deficiency

The most common nutrient deficiency worldwide is iron deficiency[1,43]. In the Western world, the prevalence of iron deficiency anaemia (IDA) is about 15 to 20% in community-dwelling populations (≥ 50 years)[4,17,19-22]. In this population IDA usually results from gastrointestinal blood loss, which is associated with esophagitis, gastritis, diverticulosis, angiodysplastic lesions, gastric or duodenal ulcers, colorectal or gastric malignancies or premalignant polyps[44-46]. In industrialised countries iron deficiency is rarely caused by insufficient dietary intake, but this is regularly encountered in the developing world[1,47].

The primary cause of vitamin B12 and/or folic acid deficiency is an inadequate dietary intake of these nutrients. Malabsorption due to gastric atrophy or pernicious anaemia may lead to vitamin B12 deficiency[48,49]. The loss of intrinsic factor, vital for the absorption of vitamin B12, will lead to the loss of ability to absorb the vitamin[50]. An uncorrected vitamin B12 deficiency may result in bone marrow failure and demyelinating nervous system disease[50]. Both intestinal malabsorption and excessive alcohol intake may cause a folic acid deficiency[48,49]. This particular deficiency is considered a risk factor for cognitive decline[51] and indicative of a poor nutritional intake[48,49]. It is also linked to raised homocysteine levels, which are associated with an increased risk for coronary heart disease, stroke and dementia[52,53] and to physical and functional decline[54].

Unknown anaemia

It is unclear whether the category 'unknown anaemia' represents one specific aetiology or a collection of several mechanisms[40]. Many possible underlying processes have been linked to or suggested for unknown anaemia in the elderly, including a blunted erythropoietic response[21,55-57], low levels of inflammatory markers[21,56,58], decreased androgen levels[58], decreased haematopoiesis[56,59-61], unrecognised renal disease[56,58], and undiagnosed or early myelodysplasia[58,59]. Despite extensive diagnostics, the proportion of unknown anaemia remains high in virtually all study populations. Adding a comprehensive haematologic evaluation does not lower this proportion[62].

Impact of anaemia on morbidity and mortality

Morbidity

Anaemia is associated with a long list of complications and increased risks for underlying conditions. An overview can be found in Figure 4.

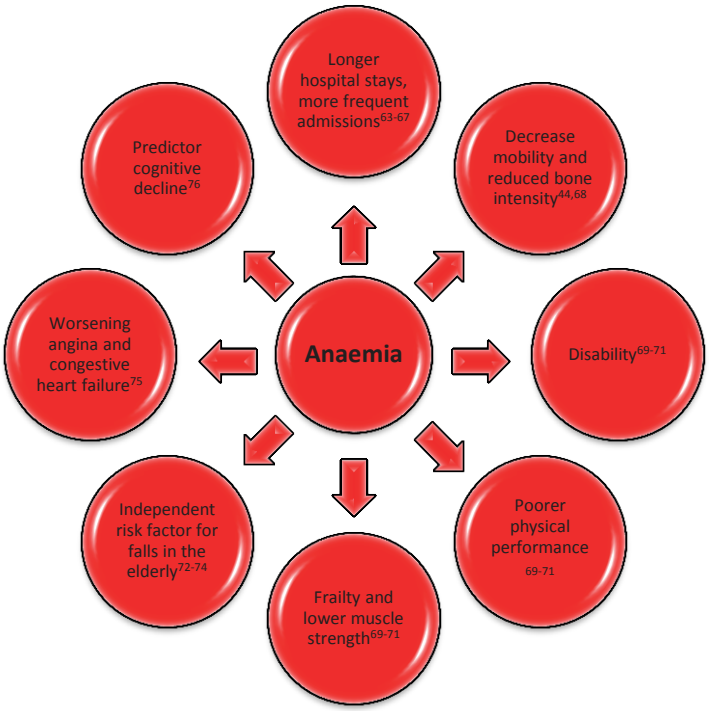


***Figure 4**, An overview of the negative effects on health associated with anaemia[44,63-76].*

A decrease in quality of life has been observed in anaemic patients, which may be due to decreased mobility[77], fatigue, changing cognitive function, depression or decreased muscle strength[44]. The association has been observed in patients diagnosed with malignancies[78,79] and chronic renal insufficiency[78,80] but also in a population of community-dwelling elderly[81].

The association of anaemia with these detrimental effects were observed in populations presenting with all levels of anaemia, from mild (Hb ≥ 10.0 g/dL) to severe (Hb < 8.0 g/dL). It could be reasoned that a more dramatically decreased Hb level will lead to more severe complaints and therefore the negative associations of anaemia would be mostly due to the severe cases. However, this is contradicted by studies including only patients presenting with mild anaemia. Mild anaemia was found to be associated with worse

selective attention performance and disease-specific quality of life ratings, poorer physical performance and functional ability, decreased muscular strength, increased frailty risk, increased risk of physical decline, recurrent falls[69,74,81-84], significant decline in quality of life[81], impaired cognition[71], and increased risk for depression and dementia[81,85].

Mortality

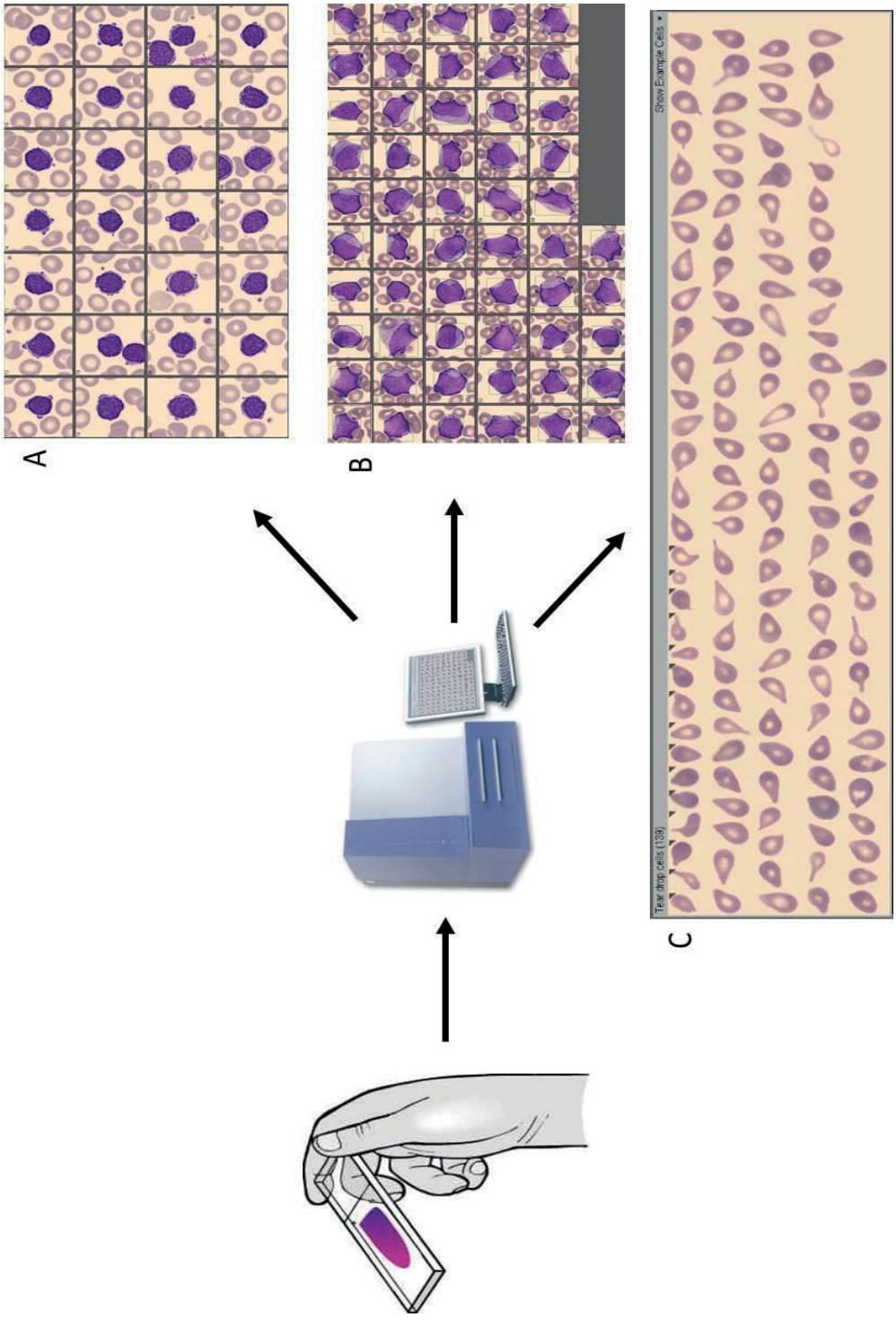
Anaemia is associated with increased all-cause mortality risk in a variety of patient cohorts. A higher risk was observed in anaemic patients diagnosed with cardiovascular diseases[64,86-90], malignancies[91], and patients undergoing haemodialysis[92,93]. Patients living in a nursing home[78,94] and the community-dwelling older population (≥ 65 years) also displayed an increased mortality risk when anaemic[65,66,84,95,96]. Again, these observations are not solely due to severe anaemia. Mild anaemia was associated with an increased risk of all-cause mortality in both the elderly (≥ 65 years)[97] and hospitalised heart failure patients[98].

A significantly increased mortality was also established in a disease-free population. This suggests that the observed effect of anaemia on mortality is, at least in part, independent of chronic or acute disease status[65].

Digital microscopy

Laboratory techniques are vital in determining the underlying aetiology of anaemia. The peripheral blood smear remains an important part of the laboratory diagnostics used for anaemia evalution[99]. Manual morphological assessment of this smear has been the gold standard for years but comes with several pitfalls. It is labour intensive, requires highly and continuously trained personnel and may be vulnerable to inter-observer variability. Digital microscopy (DM) systems have been in development for several decades and the technology has now advanced to the stage where these systems may be considered as reliable substitutes for manual assessment[100-106]. So far, the technology has mainly focused on the classification of leukocytes. Analysing these cells plays an important role in detecting a bone marrow disease. Anaemia is often the first symptom or sign of such a disease, making assessment of leukocytes on the peripheral blood smear a relevant step in anaemia analysis.

Figure 5 *(opposite page), Overview of the workflow of the digital microscope (DM). After a peripheral blood smear has been produced, it is offered to the DM which classifies the ordered number of blood cells. The system is capable of recognising a wide variety of cells. The examples in this figure include A) lymphocytes of a patient diagnosed with chronic lymphatic leukaemia (CLL) B) blast cells and C) tear drop cells.*


A
B
C
Show Example Cells
Tear drop cells (133)

Several automated morphological systems have been developed by various manufacturers since the 1970s, aided by the increasing possibilities of cameras, software and computers[107]. The DM consists of a slide scanning unit and a computer running the necessary software. The system uses a number of advanced mathematical algorithms to classify leukocytes in peripheral blood smears. Using over 250 parameters, this artificial neural network classifies each cell into one of the 11 cell classes that the system is currently capable of detecting. The parameters include shape-features, colour-features, texture-features and special features designed to capture morphological aspects of the cell such as the nucleolus or edge basophilia. This standard set of criteria is predefined by the manufacturer and cannot be altered by the operator. DM systems are capable of classifying 200 white blood cells within 2.5 minutes. The software then displays the analysed cells according to their class (e.g. neutrophils or blast cells). An operator will review the results and, if necessary, correct them before releasing the data to the laboratory information system. An overview of the workflow can be found in Figure 5.

Manual morphological assessment remains the current gold standard for the analysis of a peripheral blood smear. The validation of DM systems against this gold standard has been ongoing for the past decade. It has been shown that the classification performance of the DM is equal to manual assessment when classifying the main peripheral blood cell classes: neutrophils, lymphocytes, monocytes, eosinophils, and basophils. The detection of blast cells, essential for the correct and early diagnosis of haematological malignancies, also proved to be accurate[100-106]. Development of the technology for the classification of other cell types besides leukocytes is ongoing. DM systems can also classify erythrocytes in the peripheral blood smear[108] and nucleated cells in body fluids (cerebrospinal fluid, abdominal fluid and continuous ambulant peritoneal dialysis fluid)[109]. This is another step towards a completely automated differential of leukocytes, erythrocytes, and thrombocytes in peripheral blood, body fluids and bone marrow aspirate.

Scope of the thesis

The first part of this thesis discusses anaemia in general practice. The prevalence of the different aetiology of anaemia remains elusive. To remedy this, we analysed a large database of general practice patients newly diagnosed with anaemia. This database allowed for detailed study of the prevalence of a wide range of causes of anaemia. In addition, it supported a thorough discussion of the value of MCV in the evaluation of anaemia in general practice (**Chapter 2**). A detailed analysis of the impact of the different causes on mortality risk of anaemic general practice patients was performed. This was combined with determining the standardised mortality ratio of all-cause anaemia per five-year age group in order to demonstrate the relevance of anaemia evaluation (**Chapter 3**). Patients with macrocytic anaemia are rarely described in literature. Our population-based database allowed for a detailed description of the largest macrocytic cohort to date. The prevalence of different aetiology was determined and the impact of these causes on mortality risk was evaluated (**Chapter 4**). Lastly an analysis of the adherence of GPs to the current guidelines regarding the diagnostics and treatment of ACD and IDA was conducted (**Chapter 5** and **6**). This analysis also included the mortality risk associated with the non-application of these guidelines. These five studies comprise the first part of the thesis.

Morphological assessment of the peripheral blood smear is often part of anaemia evaluation, especially when a bone marrow disease is considered as the underlying cause. The second part of this thesis describes two steps of the validation process of DM systems, which allow for the automated and standardised assessment of peripheral blood smears. The inter-laboratory reproducibility between four DM systems was studied (**Chapter 7**), followed by a detailed analysis of a large leukocyte database, consisting of 1.4 million cells. This database was used to determine the accuracy of DM systems for a wide range of leukocyte classes, compared to manual morphological assessment, the current gold standard (**Chapter 8**).

This thesis provides reliable prevalence rates of a wide range of possible causes of anaemia and details their impact on mortality risks. More information on these topics will hopefully bring a greater awareness of the potential danger of anaemia to a patient. In addition, two steps in the validation of DM systems for use in routine practice are described. The implementation of these systems will allow for a standardised and automated differential in diagnostic laboratories.

References

1. Kassebaum NJ, Jasrasaria R, Naghavi M, et al. A systematic analysis of global anemia burden from 1990 to 2010. *Blood*. 2014;123(5):615-624.
2. Guralnik JM, Eisenstaedt RS, Ferrucci L, Klein HG, Woodman RC. Prevalence of anemia in persons 65 years and older in the united states: Evidence for a high rate of unexplained anemia. *Blood*. 2004;104(8):2263-2268.
3. Eisele L, Durig J, Broecker-Preuss M, et al. Prevalence and incidence of anemia in the german heinz nixdorf recall study. *Ann Hematol*. 2013;92(6):731-737.
4. Ania BJ, Suman VJ, Fairbanks VF, Rademacher DM, Melton LJ,3rd. Incidence of anemia in older people: An epidemiologic study in a well defined population. *J Am Geriatr Soc*. 1997;45(7):825-831.
5. Molnar MZ, Mucsi I, Macdougall IC, et al. Prevalence and management of anaemia in renal transplant recipients: Data from ten european centres. *Nephron Clin Pract*. 2011;117(2):c127-34.
6. Verbeke N, Beguin Y, Wildiers H, et al. High prevalence of anaemia and limited use of therapy in cancer patients: A belgian survey (anaemia day 2008). *Support Care Cancer*. 2012;20(1):23-28.
7. Bross MH, Soch K, Smith-Knuppel T. Anemia in older persons. *Am Fam Physician*. 2010;82(5):480-487.
8. Kaferle J, Strzoda CE. Evaluation of macrocytosis. *Am Fam Physician*. 2009;79(3):203-208.
9. Van Vranken M. Evaluation of microcytosis. *Am Fam Physician*. 2010;82(9):1117-1122.
10. Van Wijk MAM, Mel M, Muller PA, Zilverentand WGJ, Pijnenborg L, Kolnaar BGM. NHG-standaard anemie. *Huisarts & Wetenschap*. 2003;46:21-29.
11. Wintrobe MM. Anemia classification and treatment on the basis of differences in the average volume and hemoglobin content of the red corpuscles. *Archives of Internal Medicine*. 1934;54(2):256-280.
12. Seward SJ, Safran C, Marton KI, Robinson SH. Does the mean corpuscular volume help physicians evaluate hospitalized patients with anemia? *J Gen Intern Med*. 1990;5(3):187-191.
13. Bach V, Schruckmayer G, Sam I, Kemmler G, Stauder R. Prevalence and possible causes of anemia in the elderly: A cross-sectional analysis of a large european university hospital cohort. *Clin Interv Aging*. 2014;9:1187-1196.
14. Petrosyan I, Blaison G, Andres E, Federici L. Anaemia in the elderly: An aetiologic profile of a prospective cohort of 95 hospitalised patients. *Eur J Intern Med*. 2012;23(6):524-528.
15. Shavelle RM, MacKenzie R, Paculdo DR. Anemia and mortality in older persons: Does the type of anemia affect survival? *Int J Hematol*. 2012;95(3):248-256.
16. Semba RD, Ricks MO, Ferrucci L, et al. Types of anemia and mortality among older disabled women living in the community: The women's health and aging study I. *Aging Clin Exp Res*. 2007;19(4):259-264.
17. Tettamanti M, Lucca U, Gandini F, et al. Prevalence, incidence and types of mild anemia in the elderly: The "health and anemia" population-based study. *Haematologica*. 2010;95(11):1849-1856.
18. Kirkeby OJ, Fossum S, Risoe C. Anaemia in elderly patients. incidence and causes of low haemoglobin concentration in a city general practice. *Scand J Prim Health Care*. 1991;9(3):167-171.
19. Argento V, Roylance J, Skudlarska B, Dainiak N, Amoateng-Adjepong Y. Anemia prevalence in a home visit geriatric population. *J Am Med Dir Assoc*. 2008;9(6):422-426.
20. Ferrucci L, Semba RD, Guralnik JM, et al. Proinflammatory state, hepcidin, and anemia in older persons. *Blood*. 2010;115(18):3810-3816.
21. Ferrucci L, Guralnik JM, Bandinelli S, et al. Unexplained anaemia in older persons is characterised by low erythropoietin and low levels of pro-inflammatory markers. *Br J Haematol*. 2007;136(6):849-855.
22. Merlo C, Wuillemin WA. Prevalence and causes of anemia in a city general practice. *Praxis (Bern 1994)*. 2008;97(13):713-718.
23. Kahn R, Romslo I, Lamvik J. Anemia in general practice. *Scand J Clin Lab Invest Suppl*. 1990;200:41-45.
24. Roy CN. Anemia of inflammation. *Hematology Am Soc Hematol Educ Program*. 2010;2010:276-280.
25. Weiss G, Goodnough LT. Anemia of chronic disease. *N Engl J Med*. 2005;352(10):1011-1023.
26. Lipschitz D. Medical and functional consequences of anemia in the elderly. *J Am Geriatr Soc*. 2003;51(3 Suppl):S10-3.

27. Sullivan PS, Hanson DL, Chu SY, Jones JL, Ward JW. Epidemiology of anemia in human immunodeficiency virus (HIV)-infected persons: Results from the multistate adult and adolescent spectrum of HIV disease surveillance project. *Blood*. 1998;91(1):301-308.
28. van Iperen CE, van de Wiel A, Marx JJ. Acute event-related anaemia. *Br J Haematol*. 2001;115(4):739-743.
29. Ludwig H, Fritz E, Leitgeb C, Pecherstorfer M, Samonigg H, Schuster J. Prediction of response to erythropoietin treatment in chronic anemia of cancer. *Blood*. 1994;84(4):1056-1063.
30. Rizzo JD, Lichtin AE, Woolf SH, et al. Use of epoetin in patients with cancer: Evidence-based clinical practice guidelines of the american society of clinical oncology and the american society of hematology. *J Clin Oncol*. 2002;20(19):4083-4107.
31. Maury CP, Liljestrom M, Laiho K, Tiitinen S, Kaarela K, Hurme M. Tumor necrosis factor alpha, its soluble receptor I, and -308 gene promoter polymorphism in patients with rheumatoid arthritis with or without amyloidosis: Implications for the pathogenesis of nephropathy and anemia of chronic disease in reactive amyloidosis. *Arthritis Rheum*. 2003;48(11):3068-3076.
32. Gasche C, Waldhoer T, Feichtenschlager T, et al. Prediction of response to iron sucrose in inflammatory bowel disease-associated anemia. *Am J Gastroenterol*. 2001;96(8):2382-2387.
33. Wilson A, Reyes E, Ofman J. Prevalence and outcomes of anemia in inflammatory bowel disease: A systematic review of the literature. *Am J Med*. 2004;116 Suppl 7A:44S-49S.
34. Ganz T. Hepcidin, a key regulator of iron metabolism and mediator of anemia of inflammation. *Blood*. 2003;102(3):783-788.
35. Adamson JW. Renal disease and anemia in the elderly. *Semin Hematol*. 2008;45(4):235-241.
36. Cumming RG, Mitchell P, Craig JC, Knight JF. Renal impairment and anaemia in a population-based study of older people. *Intern Med J*. 2004;34(1-2):20-23.
37. Agarwal AK. Practical approach to the diagnosis and treatment of anemia associated with CKD in elderly. *J Am Med Dir Assoc*. 2006;7(9 Suppl):S7-S12; quiz S17-21.
38. Nafziger J, Pailla K, Luciani L, Andreux JP, Saint-Jean O, Casadevall N. Decreased erythropoietin responsiveness to iron deficiency anemia in the elderly. *Am J Hematol*. 1993;43(3):172-176.
39. Hsu CY, McCulloch CE, Curhan GC. Epidemiology of anemia associated with chronic renal insufficiency among adults in the united states: Results from the third national health and nutrition examination survey. *J Am Soc Nephrol*. 2002;13(2):504-510.
40. Pang WW, Schrier SL. Anemia in the elderly. *Curr Opin Hematol*. 2012;19(3):133-140.
41. Cooper AC, Mikhail A, Lethbridge MW, Kemeny DM, Macdougall IC. Increased expression of erythropoiesis inhibiting cytokines (IFN-gamma, TNF-alpha, IL-10, and IL-13) by T cells in patients exhibiting a poor response to erythropoietin therapy. *J Am Soc Nephrol*. 2003;14(7):1776-1784.
42. Zarychanski R, Houston DS. Anemia of chronic disease: A harmful disorder or an adaptive, beneficial response? *CMAJ*. 2008;179(4):333-337.
43. Killip S, Bennett JM, Chambers MD. Iron deficiency anemia. *Am Fam Physician*. 2007;75(5):671-678.
44. Andres E, Serraj K, Federici L, Vogel T, Kaltenbach G. Anemia in elderly patients: New insight into an old disorder. *Geriatr Gerontol Int*. 2013;13(3):519-527.
45. Smith DL. Anemia in the elderly. *Am Fam Physician*. 2000;62(7):1565-1572.
46. Goddard AF, James MW, McIntyre AS, Scott BB, British Society of Gastroenterology. Guidelines for the management of iron deficiency anaemia. *Gut*. 2011;60(10):1309-1316.
47. Milman N, Pedersen AN, Ovesen L, Schroll M. Iron status in 358 apparently healthy 80-year-old danish men and women: Relation to food composition and dietary and supplemental iron intake. *Ann Hematol*. 2004;83(7):423-429.
48. Andres E, Federici L, Serraj K, Kaltenbach G. Update of nutrient-deficiency anemia in elderly patients. *Eur J Intern Med*. 2008;19(7):488-493.
49. Allen LH. Causes of vitamin B12 and folate deficiency. *Food Nutr Bull*. 2008;29(2 Suppl):S20-34; discussion S35-7.
50. Stabler SP. Vitamin B12 deficiency. *New England Journal of Medicine*. 2013;368(2):149--160.

51. Kado DM, Karlamangla AS, Huang MH, et al. Homocysteine versus the vitamins folate, B6, and B12 as predictors of cognitive function and decline in older high-functioning adults: MacArthur studies of successful aging. *Am J Med*. 2005;118(2):161-167.
52. Clarke R, Smith AD, Jobst KA, Refsum H, Sutton L, Ueland PM. Folate, vitamin B12, and serum total homocysteine levels in confirmed alzheimer disease. *Arch Neurol*. 1998;55(11):1449-1455.
53. Homocysteine Studies Collaboration. Homocysteine and risk of ischemic heart disease and stroke: A meta-analysis. *JAMA*. 2002;288(16):2015-2022.
54. Ng TP, Aung KC, Feng L, Scherer SC, Yap KB. Homocysteine, folate, vitamin B-12, and physical function in older adults: Cross-sectional findings from the singapore longitudinal ageing study. *Am J Clin Nutr*. 2012;96(6):1362-1368.
55. Goodnough LT, Schrier SL. Evaluation and management of anemia in the elderly. *Am J Hematol*. 2014;89(1):88-96.
56. Merchant AA, Roy CN. Not so benign haematology: Anaemia of the elderly. *Br J Haematol*. 2012;156(2):173-185.
57. Kario K, Matsuo T, Kodama K, Nakao K, Asada R. Reduced erythropoietin secretion in senile anemia. *Am J Hematol*. 1992;41(4):252-257.
58. Makipour S, Kanapuru B, Ershler WB. Unexplained anemia in the elderly. *Semin Hematol*. 2008;45(4):250-254.
59. Steensma DP, Tefferi A. Anemia in the elderly: How should we define it, when does it matter, and what can be done? *Mayo Clin Proc*. 2007;82(8):958-966.
60. Lipschitz DA, Udupa KB, Milton KY, Thompson CO. Effect of age on hematopoiesis in man. *Blood*. 1984;63(3):502-509.
61. Rossi DJ, Bryder D, Weissman IL. Hematopoietic stem cell aging: Mechanism and consequence. *Exp Gerontol*. 2007;42(5):385-390.
62. Price EA, Mehra R, Holmes TH, Schrier SL. Anemia in older persons: Etiology and evaluation. *Blood Cells Mol Dis*. 2011;46(2):159-165.
63. Artz AS, Fergusson D, Drinka PJ, et al. Prevalence of anemia in skilled-nursing home residents. *Arch Gerontol Geriatr*. 2004;39(3):201-206.
64. Anand I, McMurray JJ, Whitmore J, et al. Anemia and its relationship to clinical outcome in heart failure. *Circulation*. 2004;110(2):149-154.
65. Penninx BW, Pahor M, Woodman RC, Guralnik JM. Anemia in old age is associated with increased mortality and hospitalization. *J Gerontol A Biol Sci Med Sci*. 2006;61(5):474-479.
66. Culleton BF, Manns BJ, Zhang J, Tonelli M, Klarenbach S, Hemmelgarn BR. Impact of anemia on hospitalization and mortality in older adults. *Blood*. 2006;107(10):3841-3846.
67. Dharmarajan TS, Pankratov A, Morris E, et al. Anemia: Its impact on hospitalizations and length of hospital stay in nursing home and community older adults. *J Am Med Dir Assoc*. 2008;9(5):354-359.
68. Patel KV, Harris TB, Faulhaber M, et al. Racial variation in the relationship of anemia with mortality and mobility disability among older adults. *Blood*. 2007;109(11):4663-4670.
69. Penninx BW, Pahor M, Cesari M, et al. Anemia is associated with disability and decreased physical performance and muscle strength in the elderly. *J Am Geriatr Soc*. 2004;52(5):719-724.
70. Ershler WB. Biological interactions of aging and anemia: A focus on cytokines. *J Am Geriatr Soc*. 2003;51(3 Suppl):S18-21.
71. Chaves PH, Carlson MC, Ferrucci L, Guralnik JM, Semba R, Fried LP. Association between mild anemia and executive function impairment in community-dwelling older women: The women's health and aging study II. *J Am Geriatr Soc*. 2006;54(9):1429-1435.
72. Dharmarajan TS, Norkus EP. Mild anemia and the risk of falls in older adults from nursing homes and the community. *J Am Med Dir Assoc*. 2004;5(6):395-400.
73. Guse CE, Porinsky R. Risk factors associated with hospitalization for unintentional falls: Wisconsin hospital discharge data for patients aged 65 and over. *WMJ*. 2003;102(4):37-42.

74. Penninx BW, Pluijm SM, Lips P, et al. Late-life anemia is associated with increased risk of recurrent falls. *J Am Geriatr Soc*. 2005;53(12):2106-2111.
75. Metivier F, Marchais SJ, Guerin AP, Pannier B, London GM. Pathophysiology of anaemia: Focus on the heart and blood vessels. *Nephrol Dial Transplant*. 2000;15 Suppl 3:14-18.
76. Denny SD, Kuchibhatla MN, Cohen HJ. Impact of anemia on mortality, cognition, and function in community-dwelling elderly. *Am J Med*. 2006;119(4):327-334.
77. Bailey RA, Reardon G, Wasserman MR, McKenzie RS, Hord RS. Association of anemia with worsened activities of daily living and health-related quality of life scores derived from the minimum data set in long-term care residents. *Health Qual Life Outcomes*. 2012;10:129-7525-10-129.
78. Landi F, Russo A, Danese P, et al. Anemia status, hemoglobin concentration, and mortality in nursing home older residents. *J Am Med Dir Assoc*. 2007;8(5):322-327.
79. Glaspy J, Bukowski R, Steinberg D, Taylor C, Tchekmedyian S, Vadhan-Raj S. Impact of therapy with epoetin alfa on clinical outcomes in patients with nonmyeloid malignancies during cancer chemotherapy in community oncology practice. procrit study group. *J Clin Oncol*. 1997;15(3):1218-1234.
80. Moreno F, Sanz-Guajardo D, Lopez-Gomez JM, Jofre R, Valderrabano F. Increasing the hematocrit has a beneficial effect on quality of life and is safe in selected hemodialysis patients. spanish cooperative renal patients quality of life study group of the spanish society of nephrology. *J Am Soc Nephrol*. 2000;11(2):335-342.
81. Thein M, Ershler WB, Artz AS, et al. Diminished quality of life and physical function in community-dwelling elderly with anemia. *Medicine (Baltimore)*. 2009;88(2):107-114.
82. Lucca U, Tettamanti M, Mosconi P, et al. Association of mild anemia with cognitive, functional, mood and quality of life outcomes in the elderly: The "health and anemia" study. *PLoS One*. 2008;3(4):e1920.
83. Penninx BW, Guralnik JM, Onder G, Ferrucci L, Wallace RB, Pahor M. Anemia and decline in physical performance among older persons. *Am J Med*. 2003;115(2):104-110.
84. Chaves PH, Semba RD, Leng SX, et al. Impact of anemia and cardiovascular disease on frailty status of community-dwelling older women: The women's health and aging studies I and II. *J Gerontol A Biol Sci Med Sci*. 2005;60(6):729-735.
85. Hong CH, Falvey C, Harris TB, et al. Anemia and risk of dementia in older adults: Findings from the health ABC study. *Neurology*. 2013;81(6):528-533.
86. da Silveira AD, Ribeiro RA, Rossini AP, et al. Association of anemia with clinical outcomes in stable coronary artery disease. *Coron Artery Dis*. 2008;19(1):21-26.
87. Dunlay SM, Weston SA, Redfield MM, Killian JM, Roger VL. Anemia and heart failure: A community study. *Am J Med*. 2008;121(8):726-732.
88. Groenveld HF, Januzzi JL, Damman K, et al. Anemia and mortality in heart failure patients a systematic review and meta-analysis. *J Am Coll Cardiol*. 2008;52(10):818-827.
89. Go AS, Yang J, Ackerson LM, et al. Hemoglobin level, chronic kidney disease, and the risks of death and hospitalization in adults with chronic heart failure: The anemia in chronic heart failure: Outcomes and resource utilization (ANCHOR) study. *Circulation*. 2006;113(23):2713-2723.
90. Maggioni AP, Opasich C, Anand I, et al. Anemia in patients with heart failure: Prevalence and prognostic role in a controlled trial and in clinical practice. *J Card Fail*. 2005;11(2):91-98.
91. Caro JJ, Salas M, Ward A, Goss G. Anemia as an independent prognostic factor for survival in patients with cancer: A systemic, quantitative review. *Cancer*. 2001;91(12):2214-2221.
92. Ofsthun N, Labrecque J, Lacson E, Keen M, Lazarus JM. The effects of higher hemoglobin levels on mortality and hospitalization in hemodialysis patients. *Kidney Int*. 2003;63(5):1908-1914.
93. Robinson BM, Joffe MM, Berns JS, Pisoni RL, Port FK, Feldman HI. Anemia and mortality in hemodialysis patients: Accounting for morbidity and treatment variables updated over time. *Kidney Int*. 2005;68(5):2323-2330.
94. Kikuchi M, Inagaki T, Shinagawa N. Five-year survival of older people with anemia: Variation with hemoglobin concentration. *J Am Geriatr Soc*. 2001;49(9):1226-1228.
95. Izaks GJ, Westendorp RG, Knook DL. The definition of anemia in older persons. *JAMA*. 1999;281(18):1714-1717.

96. Dong X, Mendes de Leon C, Artz A, Tang Y, Shah R, Evans D. A population-based study of hemoglobin, race, and mortality in elderly persons. *J Gerontol A Biol Sci Med Sci*. 2008;63(8):873-878.
97. Riva E, Tettamanti M, Mosconi P, et al. Association of mild anemia with hospitalization and mortality in the elderly: The health and anemia population-based study. *Haematologica*. 2009;94(1):22-28.
98. Teng TH, Finn J, Hung J. Mild anaemia is associated with increased all-cause mortality in heart failure. *Heart Lung Circ*. 2010;19(1):31-37.
99. Bain BJ. Diagnosis from the blood smear. *N Engl J Med*. 2005;353(5):498-507.
100. Ceelie H, Dinkelaar RB, van Gelder W. Examination of peripheral blood films using automated microscopy; evaluation of diffmaster octavia and cellavision DM96. *J Clin Pathol*. 2007;60(1):72-79.
101. Kratz A, Bengtsson HI, Casey JE, et al. Performance evaluation of the CellaVision DM96 system: WBC differentials by automated digital image analysis supported by an artificial neural network. *Am J Clin Pathol*. 2005;124(5):770-781.
102. Briggs C, Longair I, Slavik M, et al. Can automated blood film analysis replace the manual differential? an evaluation of the CellaVision DM96 automated image analysis system. *Int J Lab Hematol*. 2009;31(1):48-60.
103. Lee LH, Mansoor A, Wood B, Nelson H, Higa D, Naugler C. Performance of CellaVision DM96 in leukocyte classification. *J Pathol Inform*. 2013;4:14-3539.114205. Print 2013.
104. Cornet E, Perol JP, Troussard X. Performance evaluation and relevance of the CellaVision DM96 system in routine analysis and in patients with malignant hematological diseases. *Int J Lab Hematol*. 2008;30(6):536-542.
105. Rollins-Raval MA, Raval JS, Contis L. Experience with CellaVision DM96 for peripheral blood differentials in a large multi-center academic hospital system. *J Pathol Inform*. 2012;3:29.
106. Billard M, Lainey E, Armoogum P, Alberti C, Fenneteau O, Da Costa L. Evaluation of the CellaVision DM automated microscope in pediatrics. *Int J Lab Hematol*. 2010;32(5):530-538.
107. Tatsumi N, Pierre RV. Automated image processing. past, present, and future of blood cell morphology identification. *Clin Lab Med*. 2002;22(1):299-315, viii.
108. Egelé A, van Gelder W, Riedl J. Automated detection and classification of teardrop cells by a novel RBC module using digital imaging/microscopy.. *International Journal of Laboratory Haematology*. 2015.
109. Riedl JA, Dinkelaar RB, van Gelder W. Automated morphological analysis of cells in body fluids by the digital microscopy system DM96. *J Clin Pathol*. 2010;63(6):538-543.

PART I

Anaemia in general practice

2 |

Anaemia in general practice: an analysis of the relevance of the mean corpuscular volume for the evaluation of newly diagnosed anaemia

K.Stouten
J.A. Riedl
R.J. van Houten
J. van Rosmalen
P. Sonneveld
M-D. Levin

Submitted for publication

Abstract

Background: Anaemia is a common finding in general practice, but despite its marked prevalence, little data is available on the prevalence of the underlying causes. To determine the cause of anaemia, many physicians rely on the mean corpuscular volume (MCV) to guide further diagnostic testing. However, the exact value of MCV in general practice is unclear.

Methods: From the 1st of February 2007 until the 1st of February 2013, patients aged 50 years or older and presenting with a newly discovered anaemia to their general practitioner, were prospectively studied. Anaemia was defined as haemoglobin level below 13.7 g/dL in men and below 12.1 g/dL in women. A wide range of laboratory parameters was determined. Two independent observers established the cause or causes of anaemia, based on these parameters.

Results: A cause of anaemia could be established in 1810 (72.0%) of the 2513 included patients. Anaemia of chronic disease (29.8%), iron deficiency (18.7%) and renal anaemia (12.3%) were the most common causes. Other causes included haemoglobinopathy (0.6%) and vitamin B12 deficiency (4.2%). Strictly following the classical division of the different causes over the MCV-based classes found 28.8% of causes in the incorrect class.

Conclusion: The predictive value of MCV is not strong enough to justify excluding causes of anaemia based solely on this parameter. Existing MCV-based algorithms used for the clarification of the cause of anaemia should be reconsidered.

Introduction

Anaemia is a common finding in general practice. The prevalence of anaemia in a community-dwelling population increases from about 5% in the age group 50-64, to over 20% in people aged 85 years and older[1]. In a sample population of 65 years and older, the incidence of anaemia was found to be 12.8/1000 person-years (95% CI 9.4-17.2) in men and 10.9/1000 person years (95% CI 7.8-14.9) in women[2]. The majority of patients presents with a mild grade anaemia (Hb ≥ 10.0 g/dL)[3,4].

For many years, anaemia in the elderly was believed to be a normal consequence of aging[1,5]. However, anaemia has been shown to be associated with functional decline[5], frailty[5], increased mortality[1,6,7], increased morbidity[6,7] and decreased quality of life[7,8]. In the elderly (> 65 years), anaemia reflects both poor health and increased vulnerability to poor outcomes[5,9,10], such as longer and more frequent hospital stays[7,11-13]. These consequences are not solely associated with severe anaemia. Mild anaemia is also associated with an increased risk of hospitalisation and all-cause mortality[14]. The presence of anaemia was established as an independent predictor of survival, after adjustment for age, gender, race and chronic medical conditions[5,15].

Despite anaemia being such a frequently encountered clinical finding, information regarding the prevalence of the underlying causes is surprisingly scarce. Several studies on anaemia aetiology in the general population have been published, but in general these included relatively small patient numbers and provided a limited analysis of underlying causes[1-3,5,16-20].

The hallmark diagnostic algorithm for the evaluation of anaemia is based on the mean corpuscular volume (MCV) and encompasses an initial classification of anaemia as being microcytic, normocytic or macrocytic, with a specific set of causes listed for each class. However, the validity of this algorithm has, to the best of our knowledge, not yet been evaluated in general practice.

In this large prospective cohort study, we systematically investigated a wide range of underlying causes of anaemia in patients presenting to their general practitioner (GP). We provide reliable prevalence rates for these causes and analyse their distribution over age groups, gender, classes and types of anaemia. Furthermore, we discuss both the validity and the shortcomings of the MCV-based diagnostic algorithm.

Material and methods

Patient inclusion

This study was approved by the ethics committee of the Albert Schweitzer Hospital and included on community-dwelling men and women aged 50 years or older. These patients presented to a participating general practitioner with symptoms indicative of anaemia. In case laboratory measurements established a newly diagnosed anaemia (i.e. no known anaemia in the preceding two years, according to the laboratory information system) the patient was included in the study cohort and a standardised laboratory work-up was immediately performed. The inclusion period lasted from the 1st of February 2007 until the 1st of February 2013. The follow-up period ended on the 1st of September 2013. Anaemia was defined as haemoglobin (Hb) level below 13.7 g/dL (8.5 mmol/L, men) and below 12.1 g/dL (7.5 mmol/L, women). These haemoglobin levels constitute the cut-off values used by general practitioners in the Netherlands, as recommended by the Dutch College of General Practitioners (DCGP)[22].

Laboratory work-up

For each patient, a standardised laboratory work-up was performed, which allowed for the consideration of a broad range of causes. The following parameters were included: haemoglobin, MCV, erythrocytes, erythrocyte sedimentation rate, reticulocytes, thrombocytes, leukocytes, ferritin, transferrin, serum iron, serum vitamin B12, serum folic acid, lactate dehydrogenase (LDH), creatinin and C-reactive protein (CRP). At the discretion of the general practitioner, patients were referred to the hospital for a consultation with a medical specialist and, if necessary, additional diagnostic examinations. For these referred patients, a hospital chart review was conducted.

Classification of causes of anaemia

A classification system was developed, which included the following causes: 1) anaemia of chronic disease (ACD), 2) haemoglobinopathy, 3) haemolysis, 4) possible bone marrow disease, 5) iron deficiency (IDA), 6) vitamin B12 deficiency, 7) folic acid deficiency, 8) renal anaemia, and 9) other causes (see Table 1 for the definition of each cause). When no cause could be determined, it was classified as unknown. This system was based on the DCGP guideline[23] in use at the time of the study and on the knowledge of an experienced clinical chemist (JR) and internist (ML).

Cause	Definition
1) Anaemia of chronic disease	Ferritin > 100 µg/L, Transferrin < 2.0 g/L and/or iron < 14 µmol/L (men), < 10 µmol/L (women)
2) Haemoglobinopathy	Confirmed by DNA testing
3) Haemolysis	LDH > 450 U/L, Reticulocytes > 2.5%, Bilirubin > 17 µmol/L
4) Possible bone marrow disease	Abnormal number leukocytes and thrombocytes Reticulocytes < 2.5%
5) Iron deficiency anaemia	Ferritin < 25 µg/L (men), < 20 µg/L (women)
6) Vitamin B12 deficiency	Serum vitamin B12 < 130 pmol/L
7) Folic acid deficiency	Serum folic acid < 5 nmol/L
8) Renal anaemia	Estimated creatinin clearance (MDRD) ≤ 45 mL/min/1.73 m^2 [21]
9) Other	Reported by treating physician
10) Unknown	No cause could be determined

Table 1, *Definitions of the different causes. The cut-off values were derived from the reference intervals used in the Clinical Chemistry laboratory of the Albert Schweitzer Hospital where all tests were conducted.*

If a case classified as renal anaemia also displayed features of ACD, this case was classified as renal anaemia. ACD was only classified as a separate cause in these cases, if there was a clear indication of a concurrent infection or chronic disease. The cause of anaemia was established by two independent observers (KS and ML). In case of a discrepancy, the observers deliberated until a consensus was reached.

Definition age groups, class anaemia and type anaemia

The population was divided into four age groups: 50-64 years, 65-74 years, 75-84 years and 85+ years[1]. Cases were divided into three classes: microcytic (MCV < 80 fL), normocytic (MCV 80-100 fL) and macrocytic (MCV > 100 fL) anaemia[8,23] ; and into the types mild (Hb ≥ 10.0 g/dL), moderate (Hb 8.0-9.9 g/dL) and severe (Hb < 8.0 g/dL) anaemia[3].

Statistics

Continuous variables were described using medians, ranges and interquartile ranges (IQR), and categorical variables were described using percentages. Fisher's exact tests were used to determine the association between cause and type of anaemia, class of anaemia, age group and gender. The association between cause and age - expressed as a continuous variable- was assessed using Mann-Whitney tests, which compared the age distribution of patients diagnosed with a certain cause to the age distribution of the residual cohort. The 95% binomial proportion confidence intervals of sensitivity and specificity were calculated according to the Wilson score method.

The statistical tests were performed separately for each cause, which made it necessary to correct for multiple testing. Results for type, class and age group were considered significant when the two-sided P-values were below the Bonferroni-adjusted significance level of 0.005. Calculations were performed using Statistical Package for Social Sciences (SPSS) version 18 for Windows.

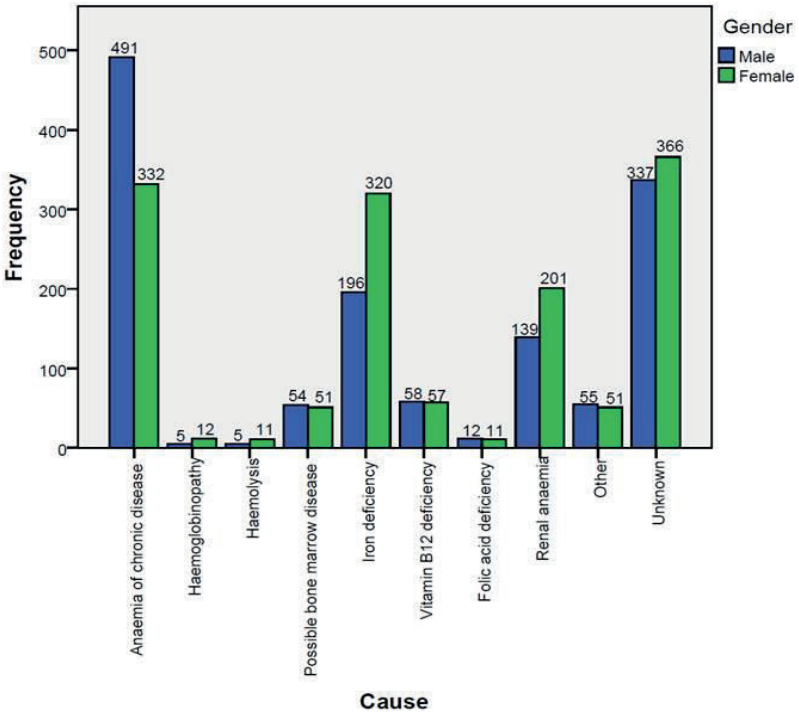


Figure 1, *Frequency of the different causes of anaemia and an overview of the frequency of each established cause divided per gender. The absolute numbers are noted above the bars. Percentages may be found in supplemental Table 1.*

Results

Patient characteristics

In a six year time period, a total of 2513 patients aged 50 years or older were included in the study, 1238 (49.3%) men and 1275 (50.7%) women. Men had a median age of 72 years (range 50-101) at the time of inclusion, women a median age of 77 years (range 50-103). An overview of patient characteristics is provided in Table 2.

Causes

Of the 2513 patients included in the study, 2284 patients (90.8%) presented with a single or unknown cause for their anaemia. A double cause was found in 211 patients (8.4%), a triple cause in 14 patients (0.6%) and a quadruple cause in 4 patients (0.2%). A total of 2764 causes, including unknown anaemia, were found in this population. ACD was found 823 times (29.8%), iron deficiency 516 times (18.7%) and renal anaemia 340 times (12.3%). Possible bone marrow disease (3.8%), vitamin B12 deficiency (4.2%) and 'other causes' (3.8%), were also found regularly (Figure 1). The cause remained unknown in 703 patients (25.4%). For a complete overview of the distribution of the causes of gender, class, age group, and type, please refer to supplemental Tables 1-4. ACD was found more often among men than women (P < 0.001), while iron deficiency and renal anaemia were more common in women (P < 0.001 and P = 0.001 respectively) (Figure 1).

Parameters	Median: IQR	Reference values
Age	75 years: 64-83	
- Men	72 years: 63-81	
- Women	77 years: 65-85	
Hemoglobin	11.8 g/dL: 11.0-12.9	
- Men	12.9 g/dL: 11.9-13.4	13.7-17.7 g/dL
- Women	11.3 g/dL: 10.5-11.8	12.1-16.1 g/dL
MCV	90 fL: 86-94	81-99 fL
Erythrocytes	4.0 10^12/L: 3.7-4.2	
- Men	4.2 10^12/L: 3.9-4.4	4.6-6.2 10^12/L
- Women	3.9 10^12/L: 3.6-4.0	4.2-5.4 10^12/L
Reticulocytes	1.0 %: 0.8-1.3	< 2.5%
Thrombocytes	272 10^9/L: 218-349	150-400 10^9/L
Leukocytes	7.0 10^9/L: 5.7-9.0	4.3-10.0 10^9/L
Ferritin	125 µg/L: 38-274	
- Men	161 µg/L: 64-334	25-250 µg/L
- Women	85 µg/L: 22-226	20-150 µg/L
Transferrin	2.4 g/L: 2.0-2.8	2.0-3.6 g/L
Iron	9.3 µmol/L: 5.9-13.8	
- Men	10.8 µmol/L: 6.1-15.3	14-28 µmol/L
- Women	8.4 µmol/L: 4.3-12.2	10-25 µmol/L
LDH	344 U/L: 300-405	< 450 U/L

Table 2, *Patient characteristics of the study population.The reference values constitute the values used in the Clinical Chemistry laboratory of the Albert Schweitzer Hospital, where all tests were conducted.*

Age and anaemia type

The number of patients in age groups 50-64, 65-74, 75-84 and 85+ were 651 (25.9%), 578 (23.0%), 745 (29.6%) and 539 (21.4%), respectively (Figure 2-A-D).

The relative prevalence of renal anaemia rose significantly with every age group, from 2.4% in age group 50-64 to 24.4% in age group 85+ ($P < 0.001$ for each comparison). Median age of patients with renal anaemia was 83.0 years (N = 340, IQR = 76.0-87.0) compared to a median age of 73 years (N = 2173, IQR = 62.5-82.0) of the rest of the cohort ($P < 0.001$).

Both haemoglobinopathy and iron deficiency were established more often in age group 50-64 compared to 65-74 ($P = 0.039$ and < 0.001), 75-84 ($P = 0.005$ and < 0.001) and 85+ ($P = 0.001$ and < 0.001). Median age for iron deficiency was 70.5 years (N = 516, IQR = 58.0-81.0) compared to a median age of 76.0 years (N = 1997, IQR = 65.0-84.0) for the residual cohort ($P < 0.001$). For patients diagnosed with haemoglobinopathy, the median age was 60.0 years (N = 17, IQR = 55.0-68.5) compared to a median age of 75.0 years (N = 2496, IQR = 64.0-83.0) of the residual cohort ($P < 0.001$).

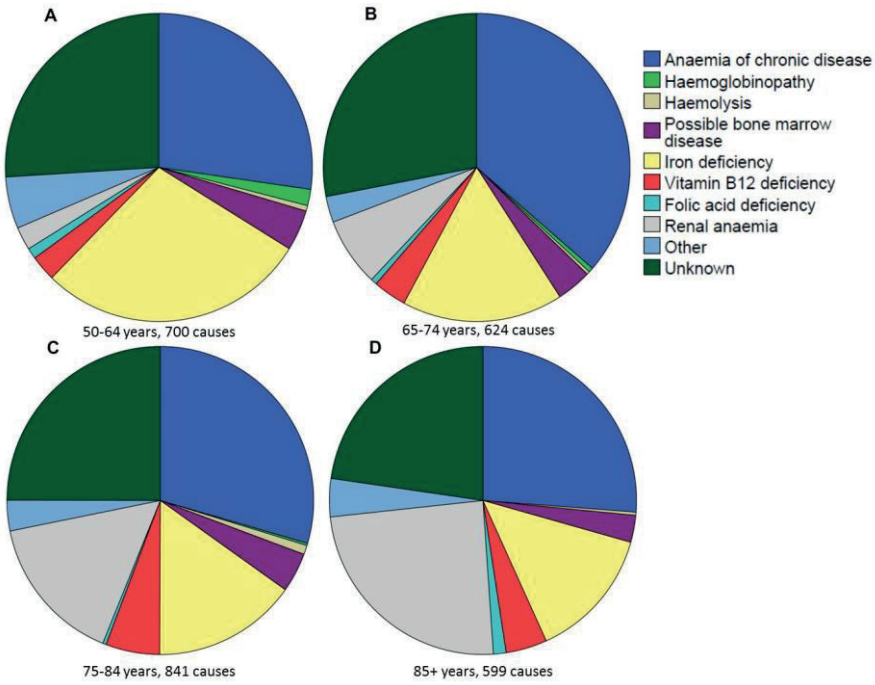


Figure 2, *Distribution of causes over the four age groups**.** Percentages and absolute numbers may be found in supplemental Table 2. A) 50-64 years (700 causes) B) 65-74 years (624 causes) C) 75-84 years (841 causes) and D) 85+ years (599 causes).*

Mild anaemia was found in a majority of 2224 patients (88.5%), moderate anaemia in 198 patients (7.9%) and severe anaemia in 91 patients (3.6%) (Figure 3- A-C). Both ACD and an unknown cause were found more often in patients with a mild anaemia (31.8% and 28.1%), compared to those with moderate (20.7% and 6.5%, P = 0.015 and P < 0.001) or severe (6.3% and 0%, both P < 0.001) anaemia. Iron deficiency was found more often in the cohort with severe anaemia (61.3%), compared to both the mild and moderate anaemia cohorts (14.6% and 39.4%, both P < 0.001).

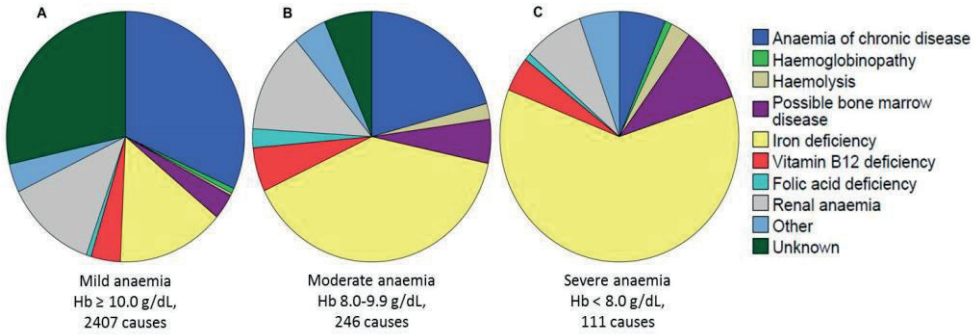


Figure 3, *Distribution of causes over the three types of anaemia**.** Percentages and absolute numbers may be found in supplemental Table 3. A) mild anaemia (Hb ≥ 10.0 g/dL, 2407 causes) B) moderate anaemia (Hb 8.0-9.9 g/dL, 246 causes) and C) severe anaemia (Hb < 8.0 g/dL, 111 causes).*

Anaemia class

Microcytic anaemia was found in 250 patients (9.9%), normocytic anaemia in 2107 patients (83.8%) and macrocytic anaemia in 156 patients (6.2%). Iron deficiency and haemoglobinopathy were more prevalent in patients presenting with a microcytic anaemia compared to both normocytic (both P < 0.001) and macrocytic (both P < 0.001) anaemia. ACD and an unknown cause were more frequent in patients presenting with normocytic anaemia as compared to both microcytic (both P < 0.001) and macrocytic (both P < 0.001) anaemia.

Haemolysis, possible bone marrow disease, vitamin B12 deficiency, folic acid deficiency and other causes can be found more often in macrocytic anaemia, compared to both microcytic (P < 0.001-0.021) and normocytic (all P < 0.001) anaemia.

Strictly following these statistical findings and classifying iron deficiency and haemoglobinopathy as microcytic, ACD and unknown as normocytic, and haemolysis, possible bone marrow disease, vitamin B12 deficiency, folic acid deficiency and other causes as macrocytic, meant that 28.8% of the causes were found in the incorrect class and 71.2% in the correct class. The relative prevalence of a cause in an incorrect class may

be small (0.3% for haemolysis within microcytic anaemia) or quite large (20.9% for ACD within macrocytic anaemia), but each cause, except haemoglobinopathy, could be found in each class of anaemia (Figure 4-A-C).

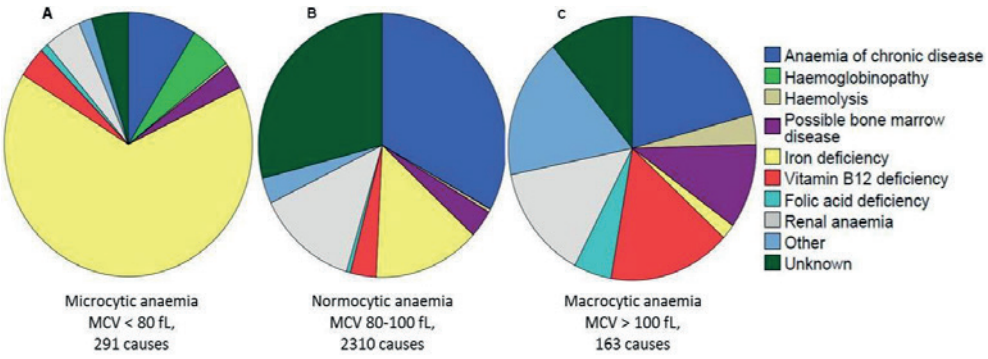


Figure 4, *Distribution of causes over the three class of anaemia**.** Percentages and absolute numbers may be found in supplemental Table 4. A) microcytic anaemia (MCV < 80 fL) B) normocytic anaemia (MCV 80-100 fL) and C) macrocytic anaemia (MCV > 100 fL).*

To further analyse the reliability of MCV for excluding causes, sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of the MCV class were calculated per established cause (Table 3). Renal anaemia was excluded from these calculations since our data did not establish a specific class for this cause. The causes as determined by the experts were considered the reference standard. For ACD, for example, patients presenting with ACD and normocytic anaemia were considered true positives, patients presenting with no ACD and normocytic anaemia were considered false positives, patients presenting with ACD and microcytic or macrocytic anaemia were considered false negatives, and patients presenting with no ACD and microcytic or macrocytic anaemia were considered true negatives.

For six causes, including the category unknown, the NPVs are considered too low to be able to use MCV as a reliable exclusionary parameter (Table 3). In addition, several causes show either a low sensitivity or specificity for MCV. The high NPV for haemoglobinopathy, haemolysis and folic acid deficiency does allow MCV to function as an exclusionary value for these causes.

Discussion

Several studies analysing causes of anaemia in general practice have been published[1-3,5,16-20], but the majority of these included only a small number of patients with anaemia. To the best of our knowledge, the cohort of anaemia patients in general practice described in this study is the largest

Cause	MCV class	Sen	95% CI	Spec	95% CI	PPV	NPV
Anaemia of chronic disease	Normocytic	92.7	90.7-94.3	20.5	18.6-22.5	36.2	85.2
Haemoglobinopathy	Microcytic	94.1	73.0-99.0	90.6	89.4-91.7	6.4	99.9
Haemolysis	Macrocytic	37.5	18.5-61.4	93.9	92.0-94.8	3.8	99.6
Possible bone marrow disease	Macrocytic	16.2	10.4-24.4	94.2	93.2-95.1	10.9	96.3
Iron deficiency anaemia	Microcytic	37.2	33.2-41.5	97.1	96.3-97.8	76.8	85.6
Vitamin B12 deficiency	Macrocytic	22.6	15.9-31.1	94.5	93.6-95.4	16.7	96.2
Folic acid deficiency	Macrocytic	34.8	18.8-55.1	94.1	93.1-94.9	5.1	99.3
Other	Macrocytic	26.4	19.0-35.5	94.7	93.7-95.5	17.9	96.7
Unknown	Normocytic	95.4	93.6-96.8	20.7	18.9-22.6	31.8	92.1

Table 3, *Statistical analysis of the reliability of MCV when excluding causes of anaemia. Sensitivity (Sen) and specificity (Spec) (with corresponding confidence intervals (CI)), positive predictive value (PPV) and negative predictive value (NPV) for each cause and corresponding class. Renal anaemia is not included in this table since statistical analysis did not show a decisive class for this cause.*

cohort analysed to date. Analysis of this cohort showed that the predictive value of MCV is not strong enough to justify using it as an exclusionary parameter.

Anaemia class

For decades, a specific set of causes has been attributed to microcytic, normocytic and macrocytic anaemia. Statistical testing on our data showed a division that generally followed the traditional grouping of causes according to MCV (i.e. iron deficiency and haemoglobinopathy are microcytic; ACD and unknown are normocytic; haemolysis, possible bone marrow disease, vitamin B12 deficiency, folic acid deficiency and other causes are macrocytic). Strictly adhering to this division resulted in 28.8% of causes found in the incorrect class. This observation may be partly explained by patients presenting with multiple causes, which were associated with different classes. For example, a patient may present with both iron and folic acid deficiency. Modern cell counters give an average when reporting MCV, so the combined microcytic (due to iron deficiency) and macrocytic (due to folic acid deficiency) erythrocytes will lead to a normocytic MCV value when measured.

Strict limits were used to establish the different nutrient deficiencies. However, for both vitamin B12 and folic acid deficiency, the values measured just above these limits (130-200 pmol/L for vitamin B12 and 5-10 nmol/L for folic acid) do not necessarily exclude a deficiency. Cases such as ACD, unknown and IDA in the macrocytic cohort, may have had an underlying vitamin B12 or folic acid deficiency, which remained undetected due to these strict limits. We may also have missed cases of IDA due to the difficulty of distinguishing between ACD and IDA when the ferritin value lies in the grey area. A serum

ferritin level ranging between 20(♀)/25(♂)-100 µg/L may be present in both causes. The criteria used in this study did not allow for the distinction between ACD and IDA when the ferritin level was found to be within this range. Cases such as vitamin B12 deficiency, folic acid deficiency and unknown in the microcytic cohort may have had an underlying iron deficiency that went unnoticed due to the set limits.

Earlier studies found the diagnostic accuracy of MCV insufficient in hospitalised patients[24,25]. Our results strongly suggest that MCV is not reliable enough to be able to exclude causes based on this value alone, when evaluating anaemia in general practice. In practice, physicians may start analysis of a potentially anaemic patient by determining the haemoglobin and MCV level, and if anaemia is established, they may be led by the MCV when deciding on further diagnostic testing. Strictly following MCV-based algorithms may result in an incorrect or incomplete diagnosis of the underlying cause or causes of anaemia.

Anaemia type and gender

ACD and an unknown cause for anaemia were shown to be associated with mild anaemia, as has been observed before[1,5]. For ACD, this association may be explained by the underlying disturbance of iron homeostasis in these patients, leading to retention of iron within the reticuloendothelial system[26]. This limits the availability of iron for the production of erythrocytes but does not completely abolish their production, resulting in a mild anaemia. Since the underlying aetiology of unknown anaemia is unclear, the association with mild anaemia is difficult to explain. It is possible that physicians are more reluctant to look for the underlying cause if a patient presents with mild anaemia compared to a patient presenting with moderate or severe anaemia. The severe anaemia cohort did not show any case of unknown anaemia. General practitioners responded to such a low haemoglobin level with a quick, sometimes immediate, referral to the hospital where the underlying cause was elucidated and consequently treated.

By far the most prevalent cause in the severe anaemia cohort was iron deficiency. This may be explained by the association of iron deficiency with occult blood loss. This loss often happens gradually and goes unnoticed for a long time. This allows the level to become severe before a patient visits his or her general practitioner, due to anaemia symptoms such as fatigue.

Shavelle *et al* (2012)[5] described a cohort similar to ours (≥ 50 years, non-institutionalised population). However, they only considered the main causes of anaemia. Their study showed a comparable prevalence for ACD (26% vs. 29.8%), nutrient deficiency (24% vs. 23.7%) and renal anaemia (11% vs. 12.3%), but did show a much higher prevalence for unknown anaemia (39% vs. 25.4%). This difference may be explained by the inclusion of

more possible causes, such as possible bone marrow disease, haemolysis and haemoglobinopathy, in our study. The decreased prevalence of unknown anaemia when including more possible causes can also be seen in the study by Tettamanti *et al* (2010)[3]. They analysed a general population aged 65 years and older from Italy and included thalassemia as a cause, in addition to the four main causes. Their study found a prevalence of 26.4% for unknown anaemia, comparable to the prevalence of 25.4% found in our cohort.

Although a broad range of causes was considered, the proportion of unknown anaemia was still high. The difficulty in distinguishing between ACD and IDA, discussed above, may partly explain this high proportion. The anaemia was classified as unknown if the ferritin level was inconclusive, unless a cause other than ACD or IDA was found. This grey area for ferritin in the diagnosis of ACD and IDA is a well-known issue[27,28]. The most recent DCGP guideline, used by GPs in the Netherlands, includes a combined diagnosis of ACD and IDA, defined as ferritin in the grey area with lowered serum iron, normal or lowered transferrin and microcytic or normocytic anaemia. The guideline recommends initially treating these patients as iron deficient[22].

A possible drawback of this study is the complete reliance on patient records, indicating the available clinical data was limited for a considerable number of patients. The laboratory tests were not repeated within the study protocol, resulting in cross-sectional data collection. However, by determining a broad range of laboratory parameters and following strict definitions for the different possible causes of anaemia, this study gives a reliable overview of a population of anaemia patients in general practice.

Conclusion

This large cohort of patients newly diagnosed with anaemia allowed for a detailed analysis of the underlying causes of anaemia in general practice. It was shown that physicians should not be solely led by the MCV value in order to exclude causes of anaemia or to decide on further diagnostic tests. This study suggests that the existing MCV-based algorithms for the evaluation of anaemia should be reconsidered.

References

1. Guralnik JM, Eisenstaedt RS, Ferrucci L, Klein HG, Woodman RC. Prevalence of anemia in persons 65 years and older in the united states: Evidence for a high rate of unexplained anemia. *Blood*. 2004;104(8):2263-2268.
2. Eisele L, Durig J, Broecker-Preuss M, et al. Prevalence and incidence of anemia in the german heinz nixdorf recall study. *Ann Hematol*. 2013;92(6):731-737.
3. Tettamanti M, Lucca U, Gandini F, et al. Prevalence, incidence and types of mild anemia in the elderly: The "health and anemia" population-based study. *Haematologica*. 2010;95(11):1849-1856.
4. Lucca U, Tettamanti M, Mosconi P, et al. Association of mild anemia with cognitive, functional, mood and quality of life outcomes in the elderly: The "health and anemia" study. *PLoS One*. 2008;3(4):e1920.
5. Shavelle RM, MacKenzie R, Paculdo DR. Anemia and mortality in older persons: Does the type of anemia affect survival? *Int J Hematol*. 2012;95(3):248-256.
6. Denny SD, Kuchibhatla MN, Cohen HJ. Impact of anemia on mortality, cognition, and function in community-dwelling elderly. *Am J Med*. 2006;119(4):327-334.
7. Andres E, Serraj K, Federici L, Vogel T, Kaltenbach G. Anemia in elderly patients: New insight into an old disorder. *Geriatr Gerontol Int*. 2013;13(3):519-527.
8. Bross MH, Soch K, Smith-Knuppel T. Anemia in older persons. *Am Fam Physician*. 2010;82(5):480-487.
9. Zakai NA, Katz R, Hirsch C, et al. A prospective study of anemia status, hemoglobin concentration, and mortality in an elderly cohort: The cardiovascular health study. *Arch Intern Med*. 2005;165(19):2214-2220.
10. Guralnik JM, Ershler WB, Schrier SL, Picozzi VJ. Anemia in the elderly: A public health crisis in hematology. *Hematology Am Soc Hematol Educ Program*. 2005:528-532.
11. Steensma DP, Tefferi A. Anemia in the elderly: How should we define it, when does it matter, and what can be done? *Mayo Clin Proc*. 2007;82(8):958-966.
12. Culleton BF, Manns BJ, Zhang J, Tonelli M, Klarenbach S, Hemmelgarn BR. Impact of anemia on hospitalization and mortality in older adults. *Blood*. 2006;107(10):3841-3846.
13. Penninx BW, Pahor M, Woodman RC, Guralnik JM. Anemia in old age is associated with increased mortality and hospitalization. *J Gerontol A Biol Sci Med Sci*. 2006;61(5):474-479.
14. Riva E, Tettamanti M, Mosconi P, et al. Association of mild anemia with hospitalization and mortality in the elderly: The health and anemia population-based study. *Haematologica*. 2009;94(1):22-28.
15. Dong X, Mendes de Leon C, Artz A, Tang Y, Shah R, Evans D. A population-based study of hemoglobin, race, and mortality in elderly persons. *J Gerontol A Biol Sci Med Sci*. 2008;63(8):873-878.
16. Semba RD, Ricks MO, Ferrucci L, et al. Types of anemia and mortality among older disabled women living in the community: The women's health and aging study I. *Aging Clin Exp Res*. 2007;19(4):259-264.
17. Kirkeby OJ, Fossum S, Risoe C. Anaemia in elderly patients. incidence and causes of low haemoglobin concentration in a city general practice. *Scand J Prim Health Care*. 1991;9(3):167-171.
18. Argento V, Roylance J, Skudlarska B, Dainiak N, Amoateng-Adjepong Y. Anemia prevalence in a home visit geriatric population. *J Am Med Dir Assoc*. 2008;9(6):422-426.
19. Ferrucci L, Guralnik JM, Bandinelli S, et al. Unexplained anaemia in older persons is characterised by low erythropoietin and low levels of pro-inflammatory markers. *Br J Haematol*. 2007;136(6):849-855.
20. Merlo C, Wuillemin WA. Prevalence and causes of anemia in a city general practice. *Praxis (Bern 1994)*. 2008;97(13):713-718.
21. Dmitrieva O, de Lusignan S, Macdougall IC, et al. Association of anaemia in primary care patients with chronic kidney disease: Cross sectional study of quality improvement in chronic kidney disease (QICKD) trial data. *BMC Nephrol*. 2013;14:24-2369-14-24.
22. NHG-werkgroep anemie. NHG standaard anemie (eerste herziening). *Huisarts & Wetenschap*. 2014;57(10):528-536.
23. Van Wijk MAM, Mel M, Muller PA, Zilverentand WGJ, Pijnenborg L, Kolnaar BGM. NHG-standaard anemie. *Huisarts & Wetenschap*. 2003;46:21-29.
24. Petrosyan I, Blaison G, Andres E, Federici L. Anaemia in the elderly: An aetiologic profile of a prospective cohort of 95 hospitalised patients. *Eur J Intern Med*. 2012;23(6):524-528.

25. Seward SJ, Safran C, Marton KI, Robinson SH. Does the mean corpuscular volume help physicians evaluate hospitalized patients with anemia? *J Gen Intern Med*. 1990;5(3):187-191.
26. Weiss G, Goodnough LT. Anemia of chronic disease. *N Engl J Med*. 2005;352(10):1011-1023.
27. Short MW, Domagalski JE. Iron deficiency anemia: Evaluation and management. *Am Fam Physician*. 2013;87(2):98-104.
28. Pasricha SR, Flecknoe-Brown SC, Allen KJ, et al. Diagnosis and management of iron deficiency anaemia: A clinical update. *Med J Aust*. 2010;193(9):525-532.

Supplemental data

Cause	Anaemia (all classes)		Men		Women	
	N	%	N	%	N	%
Anaemia of chronic disease	823	29.8	491	36.3	332	23.5
Haemoglobinopathy	17	0.6	5	0.4	12	0.8
Haemolysis	16	0.6	5	0.4	11	0.8
Possible bone marrow disease	105	3.8	54	4.0	51	3.6
Iron deficiency	516	18.7	196	14.5	320	22.7
Vitamin B12 deficiency	115	4.2	58	4.3	57	4.0
Folic acid deficiency	23	0.8	12	0.9	11	0.8
Renal anaemia	340	12.3	139	10.3	201	14.2
Other	106	3.8	55	4.1	51	3.6
Unknown	703	25.4	337	24.9	366	25.9
Total	2764	100	1352	100	1412	100

Table 1, *Distribution causes of anaemia over gender.*

Cause	Anaemia (all age groups)		50-64		65-74		75-84		85+	
	N	%	N	%	N	%	N	%	N	%
Anaemia of chronic disease	823	29.8	191	27.3	227	36.4	248	29.5	157	26.2
Haemoglobinopathy	17	0.6	12	1.7	3	0.5	2	0.2	0	0.0
Haemolysis	16	0.6	4	0.6	2	0.3	8	1.0	2	0.3
Possible bone marrow disease	105	3.8	30	4.3	23	3.7	35	4.2	17	2.8
Iron deficiency	516	18.7	199	28.4	106	17.0	128	15.2	83	13.9
Vitamin B12 deficiency	115	4.2	19	2.7	22	3.5	48	5.7	26	4.3
Folic acid deficiency	23	0.8	8	1.1	4	0.6	3	0.4	8	1.3
Renal anaemia	340	12.3	17	2.4	45	7.2	132	15.7	146	24.4
Other	106	3.8	38	5.4	17	2.7	27	3.2	24	4.0
Unknown	703	25.4	182	26.0	175	28.0	210	25.0	136	22.7
Total	2764	100	700	100	624	100	841	100	599	100

Table 2, *Distribution causes of anaemia over the different age groups*

Cause	Anaemia (all types)		Mild anaemia		Moderate anaemia		Severe anaemia	
	N	%	N	%	N	%	N	%
Anaemia of chronic disease	823	29.8	765	31.8	51	20.7	7	6.3
Haemoglobinopathy	17	0.6	16	0.7	0	0.0	1	0.9
Haemolysis	16	0.6	8	0.3	5	2.0	3	2.7
Possible bone marrow disease	105	3.8	80	3.3	14	5.7	11	9.9
Iron deficiency	516	18.7	351	14.6	97	39.4	68	61.3
Vitamin B12 deficiency	115	4.2	96	4.0	14	5.7	5	4.5
Folic acid deficiency	23	0.8	16	0.7	6	2.4	1	0.9
Renal anaemia	340	12.3	299	12.4	32	13.0	9	8.1
Other	106	3.8	89	3.7	11	4.5	6	5.4
Unknown	703	25.4	687	28.5	16	6.5	0	0.0
Total	2764	100	2407	100	246	100	111	100

Table 3, *Distribution causes over the different types of anaemia.*

Cause	Anaemia (all classes)		Microcytic anaemia		Normocytic anaemia		Macrocytic anaemia	
	N	%	N	%	N	%	N	%
Anaemia of chronic disease	823	29.8	26	8.9	763	33.0	34	20.9
Haemoglobinopathy	17	0.6	16	5.5	1	0.0	0	0.0
Haemolysis	16	0.6	1	0.3	9	0.4	6	3.7
Possible bone marrow disease	105	3.8	9	3.1	79	3.4	17	10.4
Iron deficiency	516	18.7	192	66.0	321	13.9	3	1.8
Vitamin B12 deficiency	115	4.2	11	3.8	78	3.4	26	16.0
Folic acid deficiency	23	0.8	3	1.0	12	0.5	8	4.9
Renal anaemia	340	12.3	14	4.8	303	13.1	23	14.1
Other	106	3.8	5	1.7	73	3.2	28	17.2
Unknown	703	25.4	14	4.8	671	29.0	18	11.0
Total	2764	100	291	100	2310	100	163	100

Table 4, *Distribution causes over the different classes of anaemia*

3

The impact of anaemia aetiology on mortality in a large cohort of general practice patients

K.Stouten
J.A. Riedl
R.J. van Houten
J. van Rosmalen
P. Sonneveld
M-D. Levin

Submitted for publication

Abstract

Anaemia is a common finding in general practice and is associated with an increased mortality. The individual influence of four main causes of anaemia on mortality risk has been assessed but information on the potential influence of less prevalent causes is lacking.

We analysed a cohort of community-dwelling patients (≥ 50 years) who presented with a newly diagnosed anaemia to their general practitioner. Anaemia was defined as a haemoglobin level below 13.7 g/dL (8.5 mmol/L, men) and below 12.1 g/dL (7.5 mmol/L, women).

The total number of included patients was 2929. With this large cohort we confirmed the mortality risk associated with anaemia of chronic disease (hazard ratio (HR) = 2.10, 95% CI 1.64-2.70) and renal anaemia (HR = 2.15, 95% CI 1.61-2.87). In addition to current knowledge, possible bone marrow disease (HR = 3.08, 95% CI 1.72-5.52), folic acid deficiency (HR = 6.89, 95% CI 2.79-17.04), and multiple causes (HR = 2.31, 95% CI 1.69-3.16) were shown to be associated with an increased mortality risk, compared to patients with an unknown cause of anaemia. Compared to the general Dutch population, an increased standardised mortality ratio was observed up to age 85 years. The impact of anaemia on mortality risk was particularly severe in the younger age groups. The association between mortality risk and anaemia decreased with age.

Introduction

Anaemia is a common condition in a community-dwelling population; the prevalence starts rising from the age of 50, from 4.4% in men and 6.8% in women in the age group 50-64 years, to 26.1% in men and 20.1% in women aged 85 years and older[1]. Anaemia has been associated with frailty, functional decline and decreased quality of life, and is an independent predictor of survival[1-5]. An increased mortality risk associated with anaemia was observed among community-dwelling elderly adults[6,7], chronic heart failure patients[8-12], haemodialysis patients[13,14] and nursing home residents[15,16]. These studies considered the influence of all-cause anaemia.

Information on how individual causes of anaemia affect mortality is scarce. To the best of our knowledge, only two studies have examined the relationship between the aetiology of anaemia and mortality, and both were carried out in the United States in a community-dwelling population. Shavelle *et al* (2012) used data from the Third National Health and Nutrition Survey (NHANES III) to study this relationship in a population aged 50 years and older[2]. The second study, conducted by Semba *et al* (2007), used information from the Women's Health and Aging Study I, which included moderate to severely disabled women, aged 65 years and older, still living at home[17]. These two studies found a significant influence of the aetiology of anaemia on mortality as compared to non-anaemic patients. However, only the main causes of anaemia were considered (anaemia of chronic disease (ACD), nutrient deficiency anaemia, renal anaemia and unexplained anaemia), which all showed a significant impact on mortality risk. There is no information on the influence of less prevalent causes of anaemia on mortality.

The present study analysed the relationship between anaemia aetiology and mortality in a population of patients presenting to their general practitioner (GP) with newly diagnosed anaemia. Besides the main causes of anaemia, we also took into account less prevalent causes such as possible bone marrow disease, haemolysis and haemoglobinopathy. In addition, iron deficiency, vitamin B12 deficiency and folic acid deficiency were included as separate causes instead of being clustered into nutrient deficiency. The impact of the severity of all-cause anaemia on mortality was also determined. To assess the influence of all-cause anaemia in this community-dwelling population, standardised mortality ratios (SMRs) were determined per five-year age group for each gender.

Material and methods

This study was approved by the ethical committee of the Albert Schweitzer Hospital, Dordrecht, the Netherlands.

Patient inclusion

The inclusion period lasted from the 1st of February 2007 until the 1st of February 2014. The follow-up period ended on the 1st of September 2014. Patients were included after presenting to one of the participating general practitioners with a newly diagnosed anaemia (i.e. no anaemia in the preceding two years). Both men and women were included when aged 50 years or older at the moment of presentation. Anaemia was defined as a haemoglobin level below 13.7 g/dL (8.5 mmol/L) for men and below 12.1 g/dL (7.5 mmol/L) for women. These haemoglobin levels constitute the cut-off values used by general practitioners in the Netherlands as recommended by the Dutch College of General Practitioners (DCGP)[18].

Classification causes and types of anaemia

To determine the cause of anaemia, a standardised laboratory work-up was performed. This work-up included haemoglobin, mean corpuscular volume (MCV), erythrocytes, erythrocyte sedimentation rate, reticulocytes, thrombocytes, leukocytes, ferritin, transferrin, serum iron, serum vitamin B12, serum folic acid, lactate dehydrogenase (LDH), creatinine and C-reactive protein (CRP). If, at the discretion of the general practitioner, the patient had been referred to the hospital, a hospital chart review was conducted.

The following causes were included: 1) anaemia of chronic disease (ACD) 2) haemoglobinopathy 3) haemolysis 4) possible bone marrow disease 5) vitamin B12 deficiency 6) iron deficiency anaemia (IDA) 7) folic acid deficiency 8) renal anaemia and 9) other. When no cause could be determined, it was classified as unknown. The definitions of the included causes are listed in Table 1. The cause of anaemia was established by two independent observers (authors KS and ML). In case of a discrepancy, the observers deliberated until a consensus was reached. A case classified as renal anaemia could also display features of ACD. In these cases, ACD was only classified as a separate cause, if there was a clear indication of a concurrent infection or chronic disease. Patients presenting with multiple causes were included as a separate group for survival analysis.

The population was divided into mild (Hb ≥ 10.0 g/dL), moderate (Hb 8.0-9.9 g/dL) and severe (Hb < 8.0 g/dL) anaemia in order to assess the influence of the severity of anaemia on mortality.

Survival data

Patients were followed until either their death or the 1st of September 2014, at which moment they were censored at the last date they were documented as alive in the hospital or laboratory information system. Dates of death were extracted from the same systems.

Causes	Definition
1) Anaemia of chronic disease	Ferritin > 100 µg/L, serum transferrin < 2.0 g/L and/or serum iron < 14 µmol/L (men), < 10 µmol/L (women)
2) Haemoglobinopathy	Confirmed by DNA testing
3) Haemolysis	LDH > 450 U/L, reticulocytes > 2.5%, bilirubin > 17 µmol/L
4) Possible bone marrow disease	Abnormal number leukocytes and thrombocytes, reticulocytes < 2.5%
5) Vitamin B12 deficiency	Serum vitamin B12 < 130 pmol/L
6) Iron deficiency	Ferritin < 25 µg/L (men), < 20 µg/L (women)
7) Folic acid deficiency	Serum folic acid < 5 nmol/L
8) Renal anaemia	Estimated creatinin clearance (MDRD) ≤ 45 mL/min/1.73 m^2 [19]
9) Other	Reported by treating physician
10) Unknown	No cause could be determined

***Table 1**, Definitions of the different causes. The cut-off values were derived from the reference intervals used in the Clinical Chemistry laboratory of the Albert Schweitzer Hospital where all tests were conducted.*

Data on the mortality of the general Dutch population by age and gender in the year 2010 was obtained from Statistics Netherlands, which collects and processes national statistics in the Netherlands.

Statistics

Continuous variables were described using medians and ranges, and categorical variables were described using percentages. A Cox proportional hazards model was used to assess the influence of anaemia aetiology on mortality. The follow-up time in the Cox model was defined as the time from diagnosis of anaemia to the date of death, date of last contact or the 1st of September 2014, whichever came first. The independent variables in the model were the cause of anaemia, severity of anaemia (mild, moderate or severe), age, and gender. Patients with an unknown cause of anaemia were used as reference group in the absence of a non-anaemic population. This category was selected as reference since its median haemoglobin level was closest to the normal range and due to its size. Mild anaemia was designated the reference group for the analysis of the relationship between mortality and severity of anaemia. The proportional hazards assumption in this model was tested by including interaction effects of the independent variables with time in a Cox model with time-dependent covariates. All variables were included in a single multivariable Cox model.

The years at risk were calculated by gender and five-year age groups to determine the mortality risk of the anaemic population. This was then compared to the mortality risk of the Dutch population in 2010. Confidence intervals of the standardised mortality ratios (SMRs) were calculated according to the method Ulm (1990)[20]. Calculations were performed using PASW statistics for Windows, version 18 (SPSS Inc, Chicago, U.S.) and

Excel for Windows (Microsoft, Redmond, WA, U.S.). All statistical tests were two-sided and used a significance level of 0.05.

Results

Causes of anaemia

A total of 2929 patients were included in the study; 1428 men (median age 72 years, range 50-101) and 1501 women (median age 77 years, range 50-103). Median follow-up was 29 months. A single cause could be established in 1843 patients, whereas multiple causes (double, triple or quadruple) were established in 260 patients. The cause remained unknown in 826 patients. An overview of the prevalence of the different causes of anaemia, the median age, and haemoglobin per gender can be found in Table 2.

The most prevalent causes in the category multiple causes were ACD (114 cases), iron deficiency (101 cases), renal anaemia (94 cases) and possible bone marrow disease (77 cases).

Survival analysis

Causes

Of the 2929 included patients, 557 (19.0%) died during the follow-up period (Table 2). No deaths occurred in the group of patients presenting with haemoglobinopathy and therefore no hazard ratio (HR) could be calculated for this cause. The highest proportion of deaths occurred in the categories folic acid deficiency (45.5%) and haemolysis (40.0%), which showed HRs of 6.89 (95% CI 2.79-17.04, P < 0.001) and 2.64 (95% CI 0.95-7.35, P = 0.063), respectively. Aside from the category haemoglobinopathy, the lowest proportion of deaths was found in patients with unknown anaemia (11.3%, reference group) and iron deficiency anaemia (12.4%, HR = 1.15, 95% CI 0.81-1.62, P = 0.435).

The HRs for the remaining causes in relation to patients diagnosed with unknown anaemia were as follows: anaemia of chronic disease (HR = 2.10, 95% CI 1.64-2.70, P < 0.001), possible bone marrow disease (HR = 3.08, 95% CI 1.72-5.52, P < 0.001), multiple causes (HR = 2.31, 95% CI 1.69-3.16, P < 0.001), renal anaemia (HR = 2.15, 95% CI 1.61-2.87, P < 0.001), other causes (HR = 1.84, 95% CI 1.01-3.35, P = 0.048), and vitamin B12 deficiency (HR = 1.55, 95% CI 0.87-2.77, P = 0.141) (Table 3). No significant violations of the proportional hazards assumption were detected.

Causes	Number (%)			Median age	
	Total	Men	Women	Men	Women
Anaemia of chronic disease	848 (29.0%)	507 (35.5%)	341 (22.7%)	72.0	77.0
Haemoglobinopathy	18 (0.6%)	5 (0.4%)	13 (0.9%)	56.0	61.0
Haemolysis	10 (0.3%)	5 (0.4%)	5 (0.3%)	64.0	80.0
Possible bone marrow	42 (1.4%)	25 (1.8%)	17 (1.1%)	66.0	77.0
Vitamin B12 deficiency	61 (2.1%)	28 (2.0%)	33 (2.2%)	79.5	75.0
Iron deficiency	499 (17.0%)	182 (12.7%)	317 (21.1%)	66.0	70.0
Folic acid deficiency	11 (0.4%)	4 (0.3%)	7 (0.5%)	72.0	76.0
Renal anaemia	290 (9.9%)	120 (8.4%)	170 (11.3%)	82.0	85.0
Other	64 (2.2%)	37 (2.6%)	27 (1.8%)	63.0	72.0
Multiple causes	260 (8.9%)	120 (8.4%)	140 (9.3%)	75.0	80.0
Unknown	826 (28.2%)	395 (27.7%)	431 (28.7%)	72.0	78.0
Total	2929	1428	1501	72.0	77.0

Table 2, *Overview of the prevalence of each cause and the corresponding median age*

Causes	Median haemoglobin (g/dL)		# of deaths
	Men	Women	Total
Anaemia of chronic disease	12.9	11.4	186 (21.9%)
Haemoglobinopathy	13.1	11.6	0 (0.0%)
Haemolysis	11.0	11.1	4 (40.0%)
Possible bone marrow disease	11.8	10.3	13 (31.0%)
Vitamin B12 deficiency	13.0	11.4	13 (21.3%)
Iron deficiency	12.2	10.8	62 (12.4%)
Folic acid deficiency	10.6	10.2	5 (45.5%)
Renal anaemia	12.6	11.3	94 (32.4%)
Other	13.1	11.4	12 (18.8%)
Multiple causes	12.3	10.6	75 (28.8%)
Unknown	13.2	11.6	93 (11.3%)
Total	12.9	11.3	557 (19.0%)

Table 2 (continued), *Overview of the median haemoglobin and number of deaths per cause.*

Severity of anaemia

A total of 2593 patients (88.5%) presented with mild anaemia, while 229 patients (7.8%) and 107 patients (3.7%) were found to have moderate and severe anaemia, respectively. The highest proportion of patients who died was found among patients with moderate anaemia (24.9%) followed by severe anaemia (22.4%) and mild anaemia (18.4%). A hazard ratio of 1.65 was found for both moderate anaemia (95% CI 1.23-2.20, P < 0.001) and severe anaemia (95% CI 1.06-2.57, P = 0.028) in relation to mild anaemia (Table 3).

	Hazard ratio	95% CI	P-value
Cause			< 0.001
Anaemia of chronic disease	2.10	1.64-2.70	< 0.001
Haemoglobinopathy	0.00	n/a	n/a
Haemolysis	2.64	0.95-7.35	0.063
Possible bone marrow disease	3.08	1.72-5.52	< 0.001
Vitamin B12 deficiency	1.55	0.87-2.77	0.141
Iron deficiency	1.15	0.81-1.62	0.435
Folic acid deficiency	6.89	2.79-17.04	< 0.001
Renal anaemia	2.15	1.61-2.87	< 0.001
Other	1.84	1.01-3.35	0.048
Multiple causes	2.31	1.69-3.16	< 0.001
Unknown		Reference	
Type			0.001
Moderate	1.65	1.23-2.20	< 0.001
Severe	1.65	1.06-2.57	0.028
Mild		Reference	
Gender			< 0.001
Female	0.7	0.6-0.8	< 0.001
Male		Reference	
Age	1.07	1.06-1.08	< 0.001

Table 3, *Results from the Cox proportional hazards model**.*** *Since no deaths occurred among patients with haemoglobinopathy, 95% CI and P-values could not be calculated for this cause.*

Comparison to Dutch population

The SMR was determined for each five-year age group per gender (Table 4). The age group 100-104 years was not analysed since data on the Dutch population older than 100 years was not available. Statistically significant SMRs higher than one were found for men starting in the age group 50-54 years and continuing to the age group 80-84 years. For women, significant ratios were established in the age groups 50-54 years, 60-64 years and from 70-74 years to 80-84 years. Above age 85 years, no significant impact of all-cause anaemia on mortality was observed.

Age group	Men		Women	
	Ratio	95% CI	Ratio	95% CI
50-54 years	5.01	1.03-14.64	5.30	1.72-12.37
55-59 years	4.40	2.01-8.36	2.96	0.81-7.58
60-64 years	4.32	2.64-6.68	3.57	1.43-7.35
65-69 years	2.91	1.95-4.17	2.18	0.88-4.50
70-74 years	1.96	1.30-2.83	2.04	1.09-3.50
75-79 years	1.69	1.23-2.25	2.09	1.46-2.91
80-84 years	1.34	1.01-1.73	1.50	1.11-1.99
85-89 years	1.24	0.94-1.62	1.03	0.79-1.33
90-94 years	1.15	0.80-1.60	0.97	0.72-1.28
95-99 years	0.78	0.34-1.54	1.00	0.66-1.45

Table 4, *SMRs by gender and age group.* Underlined SMRs *are statistically significant.*

Discussion

Long considered a harmless consequence of ageing, anaemia has been shown to be associated with frailty, functional decline, decreased quality of life and an increased mortality[6,7]. However, information on the possible influence of individual causes of anaemia on mortality is scarce. To the best of our knowledge, only two studies have analysed this potential influence and both only considered the main causes of anaemia; ACD, nutrient deficiency, renal anaemia and unknown anaemia[2,17]. In addition, these studies included a relatively small number of anaemic patients, 147[17] and 862[2], respectively, and one study included an all-female population aged 65 years and older[17]. We analysed a large cohort of general practice patients newly diagnosed with anaemia to determine the influence on mortality of both the main and less prevalent causes of anaemia. In addition, we determined SMRs, by comparing the mortality risks of the anaemic cohort and the general Dutch population.

Five categories of anaemia were shown to be associated with an increased mortality risk, namely ACD, possible bone marrow disease, folic acid deficiency, renal anaemia, and multiple causes. The considerable proportion of patients (16.4%, data not shown) who had already been diagnosed with an underlying malignancy at the moment of presentation or were consequently diagnosed, may account for the increased mortality risk found for the category ACD. An earlier study observed a higher prevalence of hypertension, congestive heart failure, and diabetes among patients with renal anaemia[2]; all three underlying conditions may explain the increased risk associated with this category. Due to the set-up of our study, co-morbidities were not registered and it was therefore not possible to corroborate these findings in our own cohort.

Both Semba *et al* (2007) and Shavelle *et al* (2012) had already established an increased mortality risk associated with ACD (1.48 and 1.69 respectively) and renal anaemia (1.99 and 1.70 respectively). These ratios are comparable to the ratios found in our population.

The cohort used by Shavelle *et al* (2012) corresponded closely with ours (community-dwelling population aged 50 years and older), although their study did control for a number of concomitant medical conditions, such as COPD and diabetes. Again, due to the set-up of our study, it was not possible to collect such detailed medical information. The results obtained from the large cohort of the current study definitely confirm the increased mortality risk associated with both ACD and renal anaemia.

The influence of folic acid deficiency, possible bone marrow disease, and multiple causes on mortality risk has not been established before. As in the category ACD, underlying malignancies were common among patients with possible bone marrow disease. Half of the patients (50.0%) in this category had already been or were consequently diagnosed with a malignancy, including diagnoses such as myelodysplastic syndrome (16.7%) and acute myeloid leukaemia (11.9%) (data not shown). The category 'multiple causes' contained a high proportion of ACD, possible bone marrow disease, and renal anaemia, which are all causes independently associated with an increased mortality risk. Folic acid deficiency was associated with the most severe mortality risk (HR=6.89). This deficiency, with or without associated anaemia, is a risk factor for cognitive decline[21] and is also indicative of a poor nutritional state[22,23]. Elevated homocysteine levels, which are associated with folic acid deficiency, have been connected to an increased risk of major medical conditions such as coronary heart disease, stroke, and dementia[24,25]. Shavelle *et al* (2012) found a hazard ratio of 2.34 for nutritional anaemia (vitamin B12, folic acid or iron deficiency). Our data suggest the increased mortality risk associated with nutritional anaemia was mostly due to the presence of folic acid deficiency, since independently iron deficiency and vitamin B12 deficiency did not significantly affect mortality risk.

SMRs were determined for each gender per five-year age group to assess the impact of all-cause anaemia on mortality in our population of general practice patients. Significantly increased SMRs were established up to the age group 80-84 years. The ratios showed a decrease with age. Especially in the younger age groups, anaemia had a severe impact on mortality risk. In the youngest age group studied, the risk was increased by more than five times for both men and women. Anaemia could be relatively easy to treat for these patients, and in case the anaemia is due to a severe underlying condition, these patients are much more capable of withstanding the treatment necessary for these conditions. The proper analysis and treatment of anaemia in these younger age groups may significantly improve mortality and possibly also other aspects of life and healthcare on which anaemia has an impact.

Our results show no impact of anaemia on mortality at age 85 years and upwards. This is in contrast with results from the Leiden 85-plus study, which found WHO-defined anaemia to be associated with an increased mortality risk in a population aged 85 years and older[26,27]. A possible explanation for this discrepancy may be the slightly different

populations. The Leiden 85-plus study included everyone aged 85 years and older in the city of Leiden. There was no exclusion based on living situation and patients living in nursing homes or long-term care facilities were included. The current study included community-dwelling patients visiting their general practitioner due to a specific complaint. Since blood tests are usually only ordered when a patient experiences certain complaints and consequently visits his or her physician, or if a patient is diagnosed with a condition, which requires regular monitoring, our population is more closely based on every day practice. The larger size of the current study cohort and its control group may also account for the observed discrepancy between studies.

Due to the effect of anaemia on other aspects of health, such as quality of life, which is an important factor in today's health care, an initial analysis of laboratory parameters is still advised in patients aged 85 years and older. A cause such as vitamin B12 deficiency may be found with only laboratory analysis and may be easily treated. However, if the initial laboratory measurements yield no apparent cause, we recommend to use any further invasive diagnostics with restraint. The risks of these invasive diagnostics may outweigh the potential benefits.

In conclusion, this large cohort of anaemic general practice patients confirmed the increased mortality risk associated with the causes ACD and renal anaemia. In addition, an increased risk was observed in patients presenting with possible bone marrow disease, folic acid deficiency and multiple causes. Especially in the younger age groups anaemia was shown to have a severe impact on mortality risk. From age 85 years and older, anaemia no longer has a significant impact on mortality and invasive diagnostics to determine the cause of anaemia should be used with restraint in this age group.

References

1. Guralnik JM, Eisenstaedt RS, Ferrucci L, Klein HG, Woodman RC. Prevalence of anemia in persons 65 years and older in the united states: Evidence for a high rate of unexplained anemia. *Blood*. 2004;104(8):2263-2268.
2. Shavelle RM, MacKenzie R, Paculdo DR. Anemia and mortality in older persons: Does the type of anemia affect survival? *Int J Hematol*. 2012;95(3):248-256.
3. Andres E, Serraj K, Federici L, Vogel T, Kaltenbach G. Anemia in elderly patients: New insight into an old disorder. *Geriatr Gerontol Int*. 2013;13(3):519-527.
4. Bross MH, Soch K, Smith-Knuppel T. Anemia in older persons. *Am Fam Physician*. 2010;82(5):480-487.
5. Denny SD, Kuchibhatla MN, Cohen HJ. Impact of anemia on mortality, cognition, and function in community-dwelling elderly. *Am J Med*. 2006;119(4):327-334.
6. Culleton BF, Manns BJ, Zhang J, Tonelli M, Klarenbach S, Hemmelgarn BR. Impact of anemia on hospitalization and mortality in older adults. *Blood*. 2006;107(10):3841-3846.
7. Riva E, Tettamanti M, Mosconi P, et al. Association of mild anemia with hospitalization and mortality in the elderly: The health and anemia population-based study. *Haematologica*. 2009;94(1):22-28.
8. Anand I, McMurray JJ, Whitmore J, et al. Anemia and its relationship to clinical outcome in heart failure. *Circulation*. 2004;110(2):149-154.
9. Dunlay SM, Weston SA, Redfield MM, Killian JM, Roger VL. Anemia and heart failure: A community study. *Am J Med*. 2008;121(8):726-732.
10. Go AS, Yang J, Ackerson LM, et al. Hemoglobin level, chronic kidney disease, and the risks of death and hospitalization in adults with chronic heart failure: The anemia in chronic heart failure: Outcomes and resource utilization (ANCHOR) study. *Circulation*. 2006;113(23):2713-2723.
11. Maggioni AP, Opasich C, Anand I, et al. Anemia in patients with heart failure: Prevalence and prognostic role in a controlled trial and in clinical practice. *J Card Fail*. 2005;11(2):91-98.
12. Teng TH, Finn J, Hung J. Mild anaemia is associated with increased all-cause mortality in heart failure. *Heart Lung Circ*. 2010;19(1):31-37.
13. Ofsthun N, Labrecque J, Lacson E, Keen M, Lazarus JM. The effects of higher hemoglobin levels on mortality and hospitalization in hemodialysis patients. *Kidney Int*. 2003;63(5):1908-1914.
14. Robinson BM, Joffe MM, Berns JS, Pisoni RL, Port FK, Feldman HI. Anemia and mortality in hemodialysis patients: Accounting for morbidity and treatment variables updated over time. *Kidney Int*. 2005;68(5):2323-2330.
15. Kikuchi M, Inagaki T, Shinagawa N. Five-year survival of older people with anemia: Variation with hemoglobin concentration. *J Am Geriatr Soc*. 2001;49(9):1226-1228.
16. Landi F, Russo A, Danese P, et al. Anemia status, hemoglobin concentration, and mortality in nursing home older residents. *J Am Med Dir Assoc*. 2007;8(5):322-327.
17. Semba RD, Ricks MO, Ferrucci L, et al. Types of anemia and mortality among older disabled women living in the community: The women's health and aging study I. *Aging Clin Exp Res*. 2007;19(4):259-264.
18. NHG-werkgroep anemie. NHG standaard anemie (eerste herziening). *Huisarts & Wetenschap*. 2014;57(10):528-536.
19. Dmitrieva O, de Lusignan S, Macdougall IC, et al. Association of anaemia in primary care patients with chronic kidney disease: Cross sectional study of quality improvement in chronic kidney disease (QICKD) trial data. *BMC Nephrol*. 2013;14:24-2369-14-24.
20. Ulm K. A simple method to calculate the confidence interval of a standardized mortality ratio (SMR). *Am J Epidemiol*. 1990;131(2):373-375.
21. Kado DM, Karlamangla AS, Huang MH, et al. Homocysteine versus the vitamins folate, B6, and B12 as predictors of cognitive function and decline in older high-functioning adults: MacArthur studies of successful aging. *Am J Med*. 2005;118(2):161-167.
22. Allen LH. Causes of vitamin B12 and folate deficiency. *Food Nutr Bull*. 2008;29(2 Suppl):S20-34; discussion S35-7.
23. Andres E, Federici L, Serraj K, Kaltenbach G. Update of nutrient-deficiency anemia in elderly patients. *Eur J Intern Med*. 2008;19(7):488-493.

24. Clarke R, Grimley Evans J, Schneede J, et al. Vitamin B12 and folate deficiency in later life. *Age Ageing*. 2004;33(1):34-41.
25. Ng TP, Aung KC, Feng L, Scherer SC, Yap KB. Homocysteine, folate, vitamin B-12, and physical function in older adults: Cross-sectional findings from the singapore longitudinal ageing study. *Am J Clin Nutr*. 2012;96(6):1362-1368.
26. Izaks GJ, Westendorp RG, Knook DL. The definition of anemia in older persons. *JAMA*. 1999;281(18):1714-1717.
27. den Elzen WP, Willems JM, Westendorp RG, de Craen AJ, Assendelft WJ, Gussekloo J. Effect of anemia and comorbidity on functional status and mortality in old age: Results from the leiden 85-plus study. *CMAJ*. 2009;181(3-4):151-157.

4

Macrocytic anaemia in general practice: factors influencing diagnosis and prognosis

K. Stouten
J.A. Riedl
J. Droogendijk
R. Castel
J. van Rosmalen
R.J. van Houten
P. Berendes
P. Sonneveld
M-D. Levin

Submitted for publication

Abstract

Background: Macrocytic anaemia (MCV ≥ 100 fL) is a relatively common finding in general practice. However, literature on the prevalence of the different causes in this population is limited.

Objective: To determine the prevalence of macrocytic anaemia and its aetiology in a general practice population and analyse the potential effect of the different causes on survival.

Methods: Over an eight year time period, patients aged 50 years or older and presenting to their general practitioner with a newly diagnosed anaemia were included in the study. A broad range of laboratory tests was performed for each patient. The cause of anaemia was determined by two independent observers based on the laboratory results.

Results: Of the 3324 included patients, 249 (7.5%) displayed a macrocytic anaemia and were subsequently analysed. An underlying explanation could be established in 204 patients (81.9%) with 27 patients (13.2%) displaying multiple causes. Classic aetiology (i.e. alcohol abuse, vitamin B12/folic acid deficiency, haemolysis and possible bone marrow disease) was found in 115 patients. Alternative causes (i.e. anaemia of chronic disease, iron deficiency, renal anaemia and other causes) were encountered in 101 patients.

No significant influence of the different causes on survival was observed with multivariate analysis.

Conclusion: In addition to classic explanations for macrocytosis, alternative causes are frequently encountered in patients diagnosed with macrocytic anaemia in general practice.

Background

Anaemia is regularly encountered in general practice and hospital settings. In a community dwelling population, the prevalence of anaemia starts increasing after age 50, starting from 4.4% in men and 6.8% in women in the age group 50-64 years and rising to 26.1% in men and 20.1% in women aged 85 years and older[1]. Anaemia has been shown to be an independent predictor of survival in a population of community-dwelling older adults and is capable of negatively influencing quality of life[2,3].

Macrocytic anaemia (MCV ≥ 100 fL) is a relatively common finding. Prevalence estimates for macrocytosis range from 1.7% to 3.9%. About 40% of patients presenting with macrocytosis display an associated anaemia[4,5]. Classic causes of macrocytosis include nutrient deficiencies (i.e. vitamin B12 and folic acid), alcohol abuse, liver disease, haemolysis, medication and primary bone marrow disease (e.g. myelodysplastic syndrome (MDS))[3-6]. Alternative causes include iron deficiency anaemia (IDA), anaemia of chronic disease (ACD) and renal anaemia, which are found with moderate or high prevalence in anaemia studies. However, the prevalence of these causes in patients with macrocytic anaemia remains undetermined.

A prospective cohort study was set up to systematically evaluate patients presenting with anaemia to their general practitioner. Since literature on macrocytic anaemia in general practice is limited, this study cohort was used to clarify the prevalence of macrocytic anaemia and its aetiology in newly diagnosed anaemia patients in general practice. Factors influencing the underlying causes and the potential influence of the different causes on survival in a macrocytic anaemia population were also analysed.

Methods

Patient inclusion

This study was approved by the ethical committee of the Albert Schweitzer Hospital. Patients were included after presenting to one of the participating general practitioners with a newly diagnosed anaemia (i.e. no diagnosed anaemia in the preceding two years) between the 1st of February 2007 and the 1st of February 2015. The follow-up period ended on the 1st of May 2015. Both men and women aged 50 years or older at the moment of presentation were included. Anaemia was defined as haemoglobin level below 13.7 g/dL (8.5 mmol/L) for men and below 12.1 g/dL (7.5 mmol/L) for women. These haemoglobin levels constitute the cut-off values recommended by the Dutch College of General Practitioners (DCGP)[7].

Cause	Definition
1) Vitamin B12 deficiency	Vitamin B12 < 130 pmol/L
2) Folic acid deficiency	Folic acid < 5 nmol/L
3) Possible bone marrow disease	Abnormal number leukocytes and thrombocytes Reticulocytes < 2.5%
4) Haemolysis	Raised LDH, reticulocytes > 2.5%, bilirubin > 17 µmol/L
5) Documented alcohol abuse	Reported by treating physician
6) Anaemia of chronic disease (ACD)	Ferritin > 100 µg/L, serum transferrin < 2.0 g/L and/or serum iron < 14 µmol/L (men), < 10 µmol/L (women)
7) Iron deficiency anaemia (IDA)	Ferritin < 25 µg/L (men), < 20 µg/L (women)
8) Renal anaemia	Estimated creatinin clearance (MDRD) ≤ 45 mL/min/1.73 m^2 [8]
9) Other	Reported by treating physician

Table 1, *Definitions of the different causes found in the macrocytic cohort. These cut-off values were derived from the reference intervals used in the Clinical Chemistry laboratory of the Albert Schweitzer Hospital where all tests were conducted.*

Laboratory work-up

For each patient, a standardised laboratory work-up was performed, including haemoglobin, MCV, erythrocytes, erythrocyte sedimentation rate, reticulocytes, thrombocytes, leukocytes, ferritin, transferrin, serum iron, serum vitamin B12, serum folic acid, gamma GT, LDH, creatinin and CRP. This work-up allowed for the consideration of a broad range of causes of anaemia. At the discretion of the general practitioner, patients were referred to the hospital for additional diagnostic testing. For these patients, a hospital chart review was conducted and any additional examinations were analysed.

Classification of causes of macrocytic anaemia

A classification system was developed, which included the following causes: 1) vitamin B12 deficiency 2) folic acid deficiency 3) possible bone marrow disease 4) haemolysis 5) documented alcohol abuse 6) anaemia of chronic disease (ACD) 7) iron deficiency anaemia (IDA) 8) renal anaemia and 9) other. See Table 1 for the definition of each cause. If no cause could be determined, it was classified as unknown.

Patients diagnosed with renal anaemia also frequently displayed features of ACD. These features were considered to be due to the present renal anaemia, and therefore, ACD was not classified as a separate cause in these cases. The cause of anaemia was established by two independent observers (authors KS and ML). In case of discordance, the observers deliberated until a consensus was reached.

The possible influence of the patient's age group (50-64 years, 65-74 years, 75-84 years and 85+ years) and gender on the diagnosed cause of macrocytic anaemia was also

analysed. Vitamin B12 deficiency, folic acid deficiency and iron deficiency were combined into the cause 'nutrient deficiency' for analysis.

Factors influencing survival

Survival data was extracted from the hospital and laboratory information systems. Patients were followed until either their deaths, or until the 1st of May 2015, at which moment they were censored at the last date they were documented as alive in the hospital or laboratory information system.

Patients were classified into six groups for survival analysis; nutrient deficiency (vitamin B12 deficiency, folic acid deficiency and iron deficiency), ACD, renal anaemia, multiple causes (patients who presented with a double or triple cause, except those presenting with only nutrient deficiencies), other (haemolysis, possible bone marrow disease, documented alcohol abuse and other) and unknown.

Statistics

Continuous variables were described using medians and interquartile ranges (IQR), and categorical variables were described using percentages. Fisher's exact tests were used to compare the relative prevalence (among all patients with macrocytic anaemia) of causes of anaemia between men and women and between different age groups. These tests were performed separately for each cause of anaemia (vitamin B12, folic acid and iron deficiency combined into nutrient deficiency) and for each pair of age groups. Kaplan-Meier analysis and the log rank test were used for an univariate analysis of the association between causes of anaemia and survival. A Cox proportional hazards-model correcting for the cause of anaemia, age (as a continuous variable), gender, and haemoglobin was used for a multivariate analysis of survival. The proportional hazards assumption in this model was tested by including interaction effects of the independent variables with time in a Cox model with time-dependent covariates. To account for the effects of multiple testing, a Bonferroni-adjusted significance level of 0.006 was used for Fisher's exact tests and a level of 0.008 was used for log-rank tests. For all other tests, a two-sided significance level of 0.05 was used. Calculations were performed using Statistical Package for Social Sciences (SPSS) version 18 for Windows.

Results

Over the span of eight years, a total of 3324 patients with newly diagnosed anaemia were included in the study, of whom 249 (7.5%) patients presented with a macrocytic anaemia; 140 men (median age 75 years, IQR= 63-82 years) and 109 women (median age 80 years, IQR=72.5-87 years). An overview of the patient characteristics can be found in Table 2.

A single cause of anaemia was established in 177 patients (71.1%). A double cause was established in 26 patients (10.4%) and a triple cause in one patient (0.4%). A total number of 232 causes could be established in these 204 patients, while the cause remained unknown in 45 patients (18.1%).

Parameters	Median: IQR	Reference values
Age	78.0 years: 65.0-85.0	
- Men	75.0 years: 63.0-82.0	
- Women	80.0 years: 72.5-87.0	
Haemoglobin	11.8 g/dL: 10.5-12.9	
- Men	12.9 g/dL: 11.3-13.2	13.7-17.7 g/dL
- Women	11.3 g/dL: 10.2-11.8	12.1-16.1 g/dL
MCV	103 fL: 101-108	
Erythrocytes	3.5 10^12/L: 3.0-3.8	
- Men	3.8 10^12/L: 3.1-4.0	4.6-6.2 10^12/L
- Women	3.3 10^12/L: 2.9-3.6	4.2-5.4 10^12/L
Reticulocytes	1.3%: 0.9-1.8	< 2.5%
Thrombocytes	218.0 10^9/L: 166.0-272.5	150-400 10^9/L
Leukocytes	6.7 10^9/L: 5.0-8.7	4.3-10.0 10^9/L
Ferritin	225.0 µg/L: 131.5-464.0	
- Men	250.5 µg/L: 137.0-557.8	25-250 µg/L
- Women	197.0 µg/L: 107.0-385.0	20-150 µg/L
Creatinin	84.0 µmol/L: 70.0-105.5	
- Men	88.0 µmol/L: 72.5-105.0	59-104 µmol/L
- Women	76.5 µmol/L: 62.0-106.0	45-84 µmol/L
Gamma GT	27.5 U/L: 16.0-112.5	
- Men	29.5 U/L: 18.0-168.5	< 50 U/L
- Women	23.0 U/L: 14.0-95.0	< 35 U/L

Table 2, *Patients characteristics of 249 patients with macrocytic anaemia, the interquartile range (IQR) is denoted by the first and third quartile.*

Classic causes

In this cohort, 115 patients (46.2%) presented with a total of 129 classic causes of macrocytic anaemia. Vitamin B12 deficiency was found 46 times and folic acid deficiency 16 times. Documented alcohol abuse was established 31 times, haemolysis 9 times and possible bone marrow disease 27 times (Figure 1). Bone marrow examinations performed in this last category revealed seven cases of myelodysplastic syndrome (MDS), six cases of acute myeloid leukaemia, two cases of chronic lymphoid leukaemia and two cases of multiple myeloma. In one patient, no pathology could be found. In six patients with possible bone marrow disease, no further examinations were performed due to their advanced age. In three patients with possible bone marrow disease, the abnormal

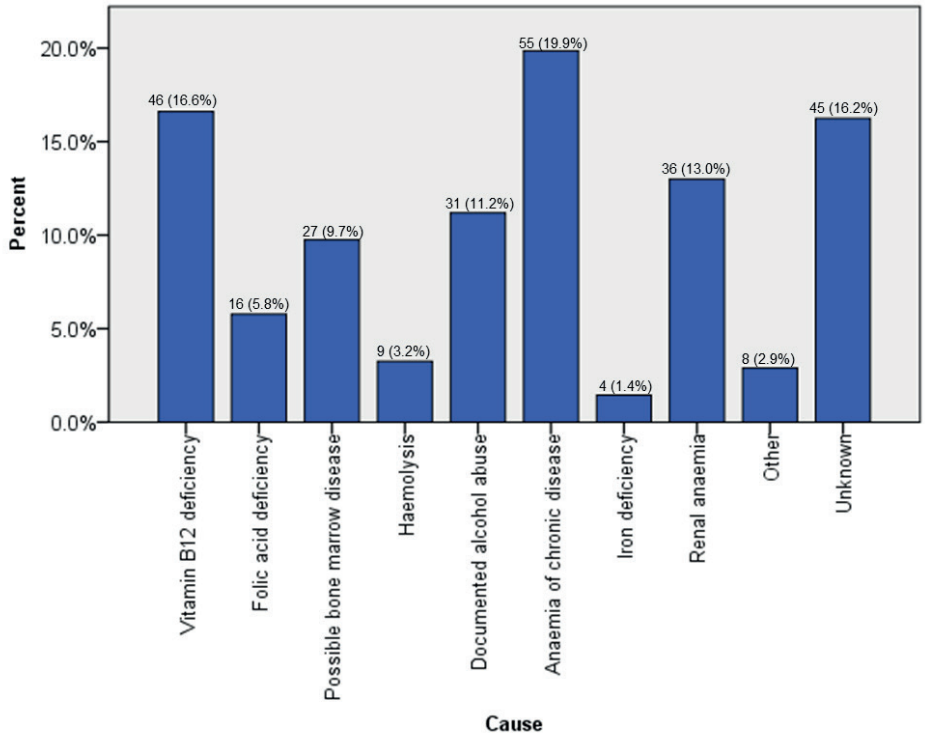


Figure 1, *An overview of the frequency of each established cause in a macrocytic population. For each patient the cause or causes were diagnosed by two independent observers.*

leukocytes and thrombocytes counts were deemed to be related to the concurrent nutrient deficiency.

Alternative causes

A total of 103 alternative causes of macrocytic anaemia were found in 101 patients (40.6%). IDA was encountered 4 times and ACD was found 55 times. Renal anaemia was established 36 times and other causes 8 times (Figure 1). This last category included three patients presenting with gastrointestinal bleeding, and three patients diagnosed with hypothyroidism. One patient was diagnosed with liver cirrhosis and one patient presented shortly after undergoing surgery.

Unknown cause of anaemia

Forty-five patients presented with an unknown cause for their anaemia. Two patients underwent a bone marrow examination in an attempt to elucidate the underlying cause.

One patient was subsequently diagnosed with MDS. For the second patient, the cause of anaemia remained unknown.

Factors influencing diagnosis

Age

58, 40, 85 and 66 patients were found in the age groups 50-64 years, 65-74 years, 75-84 years and 85+ years, respectively. Documented alcohol abuse (Figure 2-A) was most often found in the age group 50-64 years (24 of the 31 cases (77.4%)). Significant differences in prevalence of this cause were found between age group 50-64 years and the other three groups ($P < 0.001$ for all comparisons). Renal anaemia (Figure 2-B) showed a rising prevalence per age group from 1 case in 50-64 years (1.7%) to 19 cases in 85+ years (28.8%). For this cause, a significant difference was found between age groups 50-64 years and 85+ years ($P < 0.001$).

Gender

Renal anaemia was diagnosed more often in women compared to men, in 22 of 109 (20.2%) and 14 of 140 (10.0%) patients, respectively, but this difference was not significant ($P = 0.019$). No other distinct observations were made when analysing the influence of gender on the cause of anaemia.

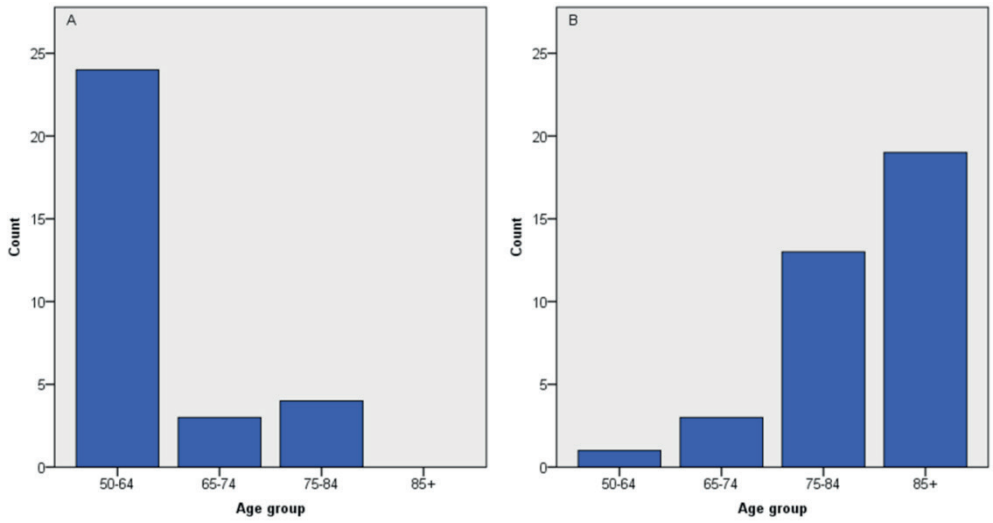


Figure 2, *Number of cases per age group for the causes documented alcohol abuse (A) and renal anaemia (B)*

Factors influencing survival

The median follow-up period lasted 25 months (95% CI 20-28 months). The Kaplan-Meier estimate of five-year survival rate from the moment of inclusion was 55.4% (95% CI 46.4-64.4) for the overall population, with a rate of 57.2% (95% CI 46.0-68.4) for men and a rate of 52.5% (95% CI 37.2-67.8) for women. Five-year survival rates were also determined for the six groups used for survival analysis: nutrient deficiency (47.9%, 95% CI 27.1-68.7), anaemia of chronic disease (56.1%, 95% CI 38.3-73.9), renal anaemia (36.6%, 95% CI 10.1-63.1), other causes (62.7%, 95% CI 47.0-78.4), multiple causes (58.8%, 95% CI 10.6-107.0) and unknown (65.4%, 95% CI 43.4-87.4) (Figure 3).

The possible influence of the six main causes on survival after entry into the study was analysed using a Cox proportional hazards model, correcting for age, gender and haemoglobin level. The group 'unknown' was selected as reference since the highest five-year survival rate was observed in this group. However, no significant influence of anaemia on survival could be found. No significant violations of the proportional hazards assumption were detected.

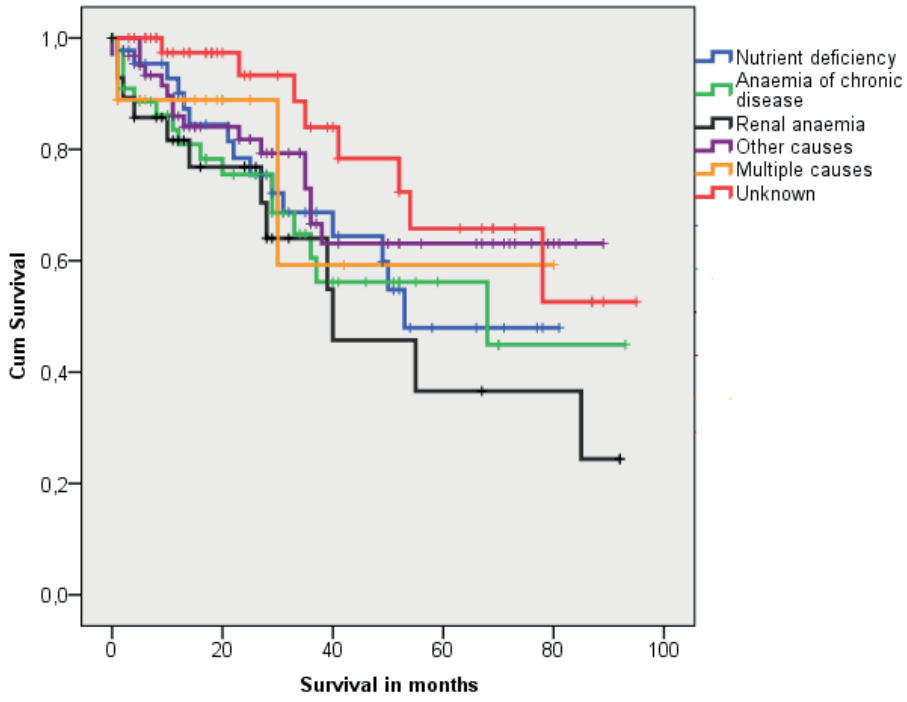


Figure 3, *Kaplan-Meier curves of the survival from the moment of inclusion for the six main causes included in survival analysis.*

Discussion and conclusions

To determine the prevalence of macrocytic anaemia and its causes in general practice, the macrocytic patients of a unique cohort of community-dwelling patients with newly diagnosed anaemia were analysed. Both classic and alternative causes of macrocytic anaemia were regularly established in this cohort. To the best of our knowledge, this is the largest study of macrocytic anaemia patients in general practice to date. Due to the set-up of the study, it relied completely on patient records.

Several algorithms have been developed to aid in diagnosing the underlying cause of macrocytic anaemia[3,9-11]. ACD, IDA and renal anaemia are rarely considered as causes of macrocytic anaemia by these algorithms, since these causes are usually reported in patients with a microcytic (MCV ≤ 80 fL) or normocytic (80 < MCV > 100) anaemia[10,12,13]. However, all three causes were frequently encountered in this macrocytic population, which has not been demonstrated so clearly in any previous studies. It is unclear what causes this shift towards macrocytosis in these patients. In literature, Andrews (2008) mentioned a possible tendency of the erythrocytes in ACD towards macrocytosis, but did not explain this further[14]. Furthermore, patients with IDA, ACD and renal anaemia were, on average, only mildly macrocytic compared to patients diagnosed with classical causes of macrocytosis, such as nutrient deficiency, alcohol abuse and possible bone marrow disease (data not shown).

For the purpose of this study, a strict limit was used when establishing the presence of vitamin B12 or folic acid deficiency. However, values measured just above these limits (range 131-200 pmol/L for vitamin B12 and 5-10 nmol/L for folic acid) may not necessarily exclude a deficiency. Several patients who were classified as having an ACD, IDA or renal anaemia, may have had an additional underlying deficiency of vitamin B12 or folic acid, which remained unclassified due to these strict limits. Other possible explanations for the presence of ACD, IDA and renal anaemia cases, include an undiscovered underlying alcohol abuse or undetected bone marrow disease. Certain medications can also cause macrocytosis. However, a complete record of medication was not available for each patient.

According to Younes *et al* (2013), unexplained macrocytosis may be a prelude to a bone marrow disease; 11.6% of their patients developed a bone marrow disease during the study period[5]. However, at the end of the follow-up period of this study, only 1 of the 45 patients (2.2%) presenting with unknown anaemia was diagnosed with such a disease, according to the documentation available in the hospital information system. The strict limits used for the classification of vitamin B12 and folic acid deficiency or undetected alcohol abuse, as described above, may also explain the presence of macrocytic anaemia

in the 44 patients whose cause of anaemia remained unknown (the 45th patient with unknown anaemia was subsequently diagnosed with MDS).

Renal anaemia showed a prevalence rising with age and was found significantly more often in patients older than 75 years. This may be explained by the decrease in renal function with age[15]. In addition, renal anaemia was observed more often among women. This may be due to the high proportion of women in the two highest age groups; 68.7% of the included women were over 75 years old at the time of inclusion versus 45.0% of men.

Documented alcohol abuse was found most often among those aged 50-64 years. After age 65, the prevalence began to decrease. This is consistent with data from a large-scale study aimed at determining prevalence of alcohol abuse performed in the United States[16].

The possible influence of different causes of anaemia on survival has not been studied before within a macrocytic cohort. However, no significant influence of the causes nutrient deficiency, renal anaemia, anaemia of chronic disease, other causes and multiple causes on survival was found. The median age of this population was high (78 years), which may make age too strong a confounding factor for a survival analysis.

Macrocytic anaemia was found in 7.5% of patients with a newly diagnosed anaemia in general practice. Anaemia of chronic disease, iron deficiency anaemia and renal anaemia were frequently encountered as causes in this macrocytic cohort. However, current algorithms for the diagnosis of macrocytic anaemia generally do not consider these alternative causes. The different underlying causes of macrocytic anaemia could have severe consequences should they remain undetected. Thus, a broad diagnostic work-up is recommended to completely elucidate the underlying cause of macrocytic anaemia.

References

1. Guralnik JM, Eisenstaedt RS, Ferrucci L, Klein HG, Woodman RC. Prevalence of anemia in persons 65 years and older in the united states: Evidence for a high rate of unexplained anemia. *Blood*. 2004;104(8):2263-2268.
2. Shavelle RM, MacKenzie R, Paculdo DR. Anemia and mortality in older persons: Does the type of anemia affect survival? *Int J Hematol*. 2012;95(3):248-256.
3. Bross MH, Soch K, Smith-Knuppel T. Anemia in older persons. *Am Fam Physician*. 2010;82(5):480-487.
4. Aslinia F, Mazza JJ, Yale SH. Megaloblastic anemia and other causes of macrocytosis. *Clin Med Res*. 2006;4(3):236-241.
5. Younes M, Dagher GA, Dulanto JV, Njeim M, Kuriakose P. Unexplained macrocytosis. *South Med J*. 2013;106(2):121-125.
6. Hoffbrand V, Provan D. ABC of clinical haematology. macrocytic anaemias. *BMJ*. 1997;314(7078):430-433.
7. NHG-werkgroep anemie. NHG standaard anemie (eerste herziening). *Huisarts & Wetenschap*. 2014;57(10):528-536.
8. Dmitrieva O, de Lusignan S, Macdougall IC, et al. Association of anaemia in primary care patients with chronic kidney disease: Cross sectional study of quality improvement in chronic kidney disease (QICKD) trial data. *BMC Nephrol*. 2013;14:24-2369-14-24.
9. Kaferle J, Strzoda CE. Evaluation of macrocytosis. *Am Fam Physician*. 2009;79(3):203-208.
10. Van Wijk MAM, Mel M, Muller PA, Zilverentand WGJ, Pijnenborg L, Kolnaar BGM. NHG-standaard anemie. *Huisarts & Wetenschap*. 2003;46:21-29.
11. Oosterhuis WP, van der Horst M, van Dongen K, Ulenkate HJ, Volmer M, Wulkan RW. Prospective comparison of the flow chart for laboratory investigations for anaemia from the dutch college of general practitioners' guideline 'anaemia' with a self-developed, substantive and logistical alternative flow chart. *Ned Tijdschr Geneeskd*. 2007;151(42):2326-2332.
12. Ganz T. Molecular pathogenesis of anemia of chronic disease. *Pediatr Blood Cancer*. 2006;46(5):554-557.
13. Ford J. Red blood cell morphology. *Int J Lab Hematol*. 2013;35(3):351-357.
14. Andrews NC. Forging a field: The golden age of iron biology. *Blood*. 2008;112(2):219-230.
15. McFarlane SI, Chen SC, Whaley-Connell AT, et al. Prevalence and associations of anemia of CKD: Kidney early evaluation program (KEEP) and national health and nutrition examination survey (NHANES) 1999-2004. *Am J Kidney Dis*. 2008;51(4 Suppl 2):S46-55.
16. Hasin DS, Stinson FS, Ogburn E, Grant BF. Prevalence, correlates, disability, and comorbidity of DSM-IV alcohol abuse and dependence in the united states: Results from the national epidemiologic survey on alcohol and related conditions. *Arch Gen Psychiatry*. 2007;64(7):830-842.

5

Adherence to the national guideline in patients with newly discovered anaemia of chronic disease in general practice

A. Schop
K. Stouten
J. Droogendijk
R.J. van Houten
J.A. Riedl
M-D. Levin

Submitted for publication

Abstract

Introduction: In spite of its relatively high incidence, little is known regarding the diagnosis and treatment of anaemia of chronic disease (ACD) in primary care. We set out to study all investigations and therapeutic interventions by general practitioners (GPs), and the established underlying causes of ACD, to determine the adherence to the national guideline in newly diagnosed ACD patients.

Methods: Information regarding patients with newly diagnosed ACD was obtained from the information system of the GP and the referral hospital. ACD is defined as haemoglobin below 13.7 g/dL (male) and 12.1 g/dL (female) and ferritin above 100 µg/L combined with decreased iron and/or reduced transferrin.

Results: Of 520 patients with newly diagnosed ACD presenting to 41 participating GPs, 290 (56%) could be analysed. In 226 of 290 patients (78%), an underlying cause of ACD could be established. In 193 patients (67%), additional investigations were required and the cause was apparent at the time of diagnosis in 33 patients (11%). The treatments administered were in concordance with the national Dutch guideline for 211 patients (73%). However, 39 patients (13%) received inappropriate oral iron supplementation. In relation to the reference group, survival was poorer in patients with malignancy ($P < 0.001$) and tended to be better in patients with autoimmune disease ($P = 0.05$).

Conclusions: This unique dataset on the diagnostic and therapeutic strategies of GPs in patients with newly diagnosed ACD reveals that the majority of patients were diagnosed and treated according to the national guideline, but inappropriate iron supplementation was prescribed in a substantial amount of patients.

Introduction

Anaemia is a common finding in general practice and is associated with increased mortality, physical and cognitive decline, collapse, fractures, frailty, cardiovascular events and reduced quality of life[1]. One of the most prevalent categories of anaemia is anaemia of chronic disease (ACD)[2]. ACD is caused by a wide variety of conditions, such as acute infections, chronic disease (chronic lung disease, diabetes), autoimmune disorders, acute trauma, surgical interventions, renal failure, heart failure and malignancies[3-7]. All of these conditions may lead to increased concentrations of inflammatory cytokines, which induce the production of the hormone hepcidin. This hormone blocks the release of iron from macrophages, hepatocytes and enterocytes by removing the ferroportin receptor from the cell membranes of these cells[8]. In addition, hepcidin causes a down regulation of the duodenal absorption of iron. Together, these processes result in a functional iron deficiency[8].

The prevalence of ACD has been reported to range from 13.6% to 47.2% in the elderly population (≥ 65 years)[2]. In a population of patients aged 50 years and older, a relative risk for mortality of 1.48 ($P < 0.001$) was found in patients with ACD compared to adults without anaemia[9]. Due to this impact, it is important that general practitioners (GPs) provide the best available care for patients with ACD. In order to do so, the clarification of the underlying disease, which is causing ACD, is often necessary. In the Netherlands, the Dutch College of General Practitioners (DCGP) provides a guideline for GPs on handling newly discovered ACD. This national guideline recommends performing further investigations in order to determine the underlying disease, unless a recently incurred disease can explain the ACD. Since ACD is associated with a functional iron deficiency and oral iron uptake is diminished, the guideline also states that patients with ACD should not be treated with oral iron supplementation[10]. We set out to investigate the diagnostic and therapeutic strategies that GPs employ in patients with newly discovered ACD and the survival rates of these patients. The adherence of GPs to the national guideline and the correlation of different factors, such as gender, age, symptoms, and laboratory results with adherence were also investigated.

Methods

Patient inclusion

From the 1st of February 2007 to the 1st of February 2013 all patients presenting to one of 41 GPs, who participate in a transmural anaemia intervention programme, with newly diagnosed ACD (i.e. no anaemia in the previous two years), were included. This study was approved by the internal ethics committee of the Albert Schweitzer Hospital and the participating GPs gave permission for the use of anonymous medical information.

Anaemia was defined as haemoglobin below 13.7 g/dL (8.5 mmol/L) for males and below 12.1 g/dL (7.5 mmol/L) for females. ACD was defined as ferritin above 100 µg/L combined with decreased serum iron and/or reduced transferrin (i.e. below the lower limit of the reference interval)[11]. Males aged 18 years and older, and females aged 50 years and older (in order to exclude a predominance of hypermenorrhea as the cause of anaemia in the transmural anaemia project) were included. In order to maintain equality in laboratory settings and the criteria to define ACD, all analyses were performed in the clinical chemistry laboratory of the Albert Schweitzer Hospital.

Data collection

All investigations, performed from the diagnosis of ACD to either the discovery of the underlying cause or the first outpatient visit to the referral hospital, were collected from the information systems of both the GPs and the referral hospital. In addition, the time (in weeks) from establishing the ACD to diagnosis of the underlying cause, and to death or end of follow-up were registered, too. The end of follow-up period was defined as the date of data collection from the information system.

Typical and atypical presentation

The symptoms of the patient on the day of blood collections were recorded by the GP. The symptoms were classified as atypical or typical complaints. The division was determined by experienced internists. Typical complaints consisted of abdominal complaints, rectal bleeding, (blood) vomiting, diarrhoea, oedema, renal failure, pain lower back/inguinal/lumbago, swollen lymph nodes, vaginal bleeding, heart problems, lung problems, haematuria and fever. Atypical complaints consisted of non-localised complaints such as tiredness, weight loss, joint pain, malaise, myalgia, itch, neck/headache, dizziness, and pain in the extremities. If patients had both atypical and typical complaints, the patient was recorded as having typical complaints.

Diagnostic and therapeutic strategies

All investigations ordered by GPs, such as x-ray, endoscopies and ultrasounds, were recorded, including those requested in consultation with medical specialists. However, any investigations performed after the patient was referred to a medical specialist were not included in the analysis. Prescription of oral iron supplementation was registered to help determine adherence to the DCGP guideline. Adherence to the DCGP guideline, set as the gold standard, was defined as (I) additional investigations in order to clarify the underlying illness causing ACD unless an underlying cause was already known, and (II) no oral iron supplementation.

Underlying causes of ACD

All underlying causes of ACD were registered, both those diagnosed by the GP and those diagnosed by the medical specialist. If the patient was known to also suffer from diabetes, chronic lung disease or heart failure, and no other underlying cause of ACD could be established, these diseases were assigned as the underlying cause of ACD.

Statistical analysis

The data was analysed using the Statistical Package for the Social Sciences (SPSS) for Windows, version 18. The patient population was characterised by standard descriptive statistics, such as mean with standard deviation, frequency counts, percentages and ranges. Differences in mean haemoglobin levels between two groups were tested using the independent t-test. Factors influencing the adherence to the DCGP guideline were analysed using binary logistic regression. The survival rates of patients were analysed using the Cox regression model. P-values lower than 0.05 were considered significant.

Results

Patient characteristics

A total of 520 patients presenting with ACD were identified. No records were available for 230 patients, leaving a total of 290 patients (56%) with a newly discovered ACD for analysis. Of these 290 patients, 171 were male (59%) and 119 were female (41%). Patient characteristics are shown in Table 1. Symptoms on the day of blood collection were registered in 261 patients (90%), and of these patients, 130 (50%) had atypical complains and 131 (50%) had typical complaints. In 29 patients (10%) symptoms on day of blood collection were unknown or blood was collected as part of a routine examination because of another illness.

Additional investigations by GPs

In 222 of 290 patients (77%), additional investigations were performed by the GPs after ACD was established. In 81 patients (28%), multiple investigations were performed, leading to a total of 341 investigations. In 193 patients (67%) the additional investigations led to the establishment of an underlying cause of ACD. An overview of all requested investigations and the ration to established causes of each investigation is displayed in Table 2. Patients who were referred to a medical specialist after establishment of the ACD had a mean haemoglobin level of 11.7 g/dL (range 8.1-13.5) as compared to a mean haemoglobin level of 12.0 g/dL (range 7.9-13.5) for patients who were not

	Mean ± S.D.	Reference value
Age (years):	72 ± 13.5	
- Male	68 ± 14.7	
- Female	77 ± 9.7	
Haemoglobin (g/dL):	11.9 ± 1.3	
- Male	12.5 ± 1.1	13.7-17.7
- Female	11.0 ± 1.0	12.1-16.1
Transferrin (g/dL):	1.97 ± 0.36	2.0-3.6
Ferritin (µg/L):	418 ± 352	
- Male	446 ± 335	25-250
- Female	379 ± 373	20-150
Serum iron (µmol/L):	6.6 ± 3.7	
- Male	7.1 ± 3.8	14-28
- Female	5.9 ± 3.4	10-25

***Table 1**, Characteristics of ACD patients. S.D.=standard deviation*

	Cause established/frequency (%)
Physical examination	29/29 (100)
X-ray	
- Thorax	19/83 (23)
- Abdomen	2/11 (18)
- Joint	1/9 (11)
- Sinus	1/2 (50)
Ultrasound	
- Abdomen	4/28 (14)
- Renal	1/1 (100)
CT scan	
- Abdomen/thorax	7/13 (54)
- Skull	0/1 (0)
Endoscopy	
- Full endoscopy	0/2 (0)
- Gastroscopy	1/6 (17)
- Colonoscopy	0/5 (0)
Referral	
- Internist	61/68 (90)
- Emergency room	29/32 (91)
- Pulmonologist	13/14 (93)
- Geriatrician	8/11 (73)
- Rheumatologist	9/9 (100)
- Other	8/17 (47)
Total	193/341 (57)

***Table 2**, Ratio of cause established and recorded investigations; values are numbers (%)*

referred (P = 0.064). An underlying cause of ACD could be determined in 226 of 290 patients (78%). In 193 patients (67%), the cause was established through additional investigations and in 33 patients (11%) the underlying cause was apparent at the time of diagnosis of ACD. An overview of all causes is displayed in Table 3.

Underlying causes of ACD

In 152 of 193 patients (79%) the time to the diagnosis of the underlying cause could be established. The average time was 4.6 weeks (range 0-48). In 50 of 152 patients (33%) the underlying cause was established within one week and in 92 of 152 patients (61%) the underlying cause could be established within four weeks after presentation (Supplemental data 1).

Malignancy as the underlying cause of ACD was found in 50 patients (22%), of which 25 were metastatic (50%), 11 non-metastatic (225), eight patients (16%) presented with B-cell lymphoma and in six patients (12%) no further dissemination investigations were performed (type and stage of the malignancies are described in Supplemental data 2).

	Additional investigations (N = 222)	**Underlying disease known (N = 33)**	**Total**
Underlying cause established	193 (87)	33 (100)	226 (78)
- Autoimmune disease	42 (22)	10 (30)	52 (23)
- Infection	77 (40)	0 (0)	77 (34)
- Renal failure	4 (2)	0 (0)	4 (2)
- Recent operation	3 (2)	2 (6)	5 (2)
- Malignancy	46 (24)	4 (12)	50 (22)
- Diabetes	7 (4)	12 (36)	19 (8)
- Heart failure	6 (3)	2 (6)	8 (4)
- Chronic lung disease	4 (2)	1 (3)	5 (2)
- Other causes *	4 (2)	2 (6)	6 (3)
No cause established	29 (13)	0 (0)	64 (22)

***Table 3**, underlying causes of ACD; values are numbers (%). The percentage of underlying causes is calculated from the total amount of causes established in each subgroup. In this table, 35 patients, who did not receive additional investigations and in whom no underlying cause was established, are not shown. Therefore, the total amount of patients with no cause established included 35 more cases. * Other causes such as liver cirrhosis, haematoma and alcohol abuse.*

Survival analysis

The survival of patients with newly diagnosed ACD for the four main underlying causes (autoimmune disease, infection, malignancy or other cause) is shown in Figure 1. In addition, the survival of patients, in whom no underlying cause could be established in

	Hazard ratio (95% CI)	P-value
Age	1.06 (1.03-1.08)	< 0.001
Gender	1.11 (0.70-1.74)	0.66
No additional investigations, no cause established	Reference	
Autoimmune disease	0.31 (0.09-1.02)	0.05
Infection	1.15 (0.48-2.74)	0.75
Malignancy	6.85 (3.11-15.09)	< 0.001
Other causes	1.25 (0.50-3.17)	0.63
Additional investigations, no cause established	1.35 (0.48-3.78)	0.56

***Table 4**, Multivariate analysis of the survival data of ACD patients. The multivariate analysis of the survival for the different underlying causes is performed with 'no additional investigations, no cause established' as the reference group.*

combination with or without additional investigation, is shown. Multivariate analysis demonstrated an association between survival and age (HR = 1.06, 95% CI: 1.03-1.08, P < 0.001). The survival analysis also showed a poor survival rate in patients with malignancy (HR = 6.85, 95% CI: 3.11-15.09, P < 0.001) and tended to be better in patients with autoimmune disease (HR = 0.31, 95% CI: 0.09-1.02, P = 0.05) in relation to the reference group "no additional investigations, no cause established" (Table 4).

The proportional hazard assumption showed no signification P-values for age (P = 0.79), gender (P = 0.71) and underlying cause (P = 0.70) confirming the multivariate analysis performed was appropriate for the study population.

	Odds ratio (95% CI)	P-value
Oral iron supplementation		
Age	0.99 (0.97-1.02)	0.663
Gender	1.44 (0.64-3.25)	0.375
Haemoglobin	0.53 (0.32-0.87)	0.012
Ferritin	0.998 (0.996-1.000)	0.021
DCGP guideline		
Age	0.99 (0.96-1.01)	0.186
Gender	1.05 (0.54-2.05)	0.878
Haemoglobin	0.99 (0.64-1.53)	0.973
Ferritin	1.00 (1.00-1.00)	0.005
Transferrin	0.74 (0.33-1.67)	0.468
Atypical complaints	2.91)1.22-6.96)	0.016
Typical complaints	1.81 (0.77-4.27)	0.172

***Table 5**, Factors associated with prescription of oral iron supplementation or adherence to the DCGP guideline. P-value below 0.05 is considered significant*

Oral iron supplementation

Thirty-nine patients (13%) received oral iron supplementation despite being diagnosed with ACD and showing ferritin levels above 100 µg/L. Lower haemoglobin and minimally raised ferritin levels (above 100 µg/L) are associated with a higher probability of iron supplementation, OR = 0.53 (P = 0.012) and OR = 0.998 (P = 0.021), respectively (Table 5).

GPs adherence to the DCGP guideline

In 211 of 290 patients (73%) the GP followed the DCGP guideline, consisting of additional investigations, unless a recently incurred disease can explain the ACD, in combination with no oral iron supplementation. Increased ferritin levels and atypical complaints are associated with a higher probability of the DCGP guideline being applied, OR = 1.002 (P = 0.005) and OR = 2.91 (P = 0.016), respectively (Table 5).

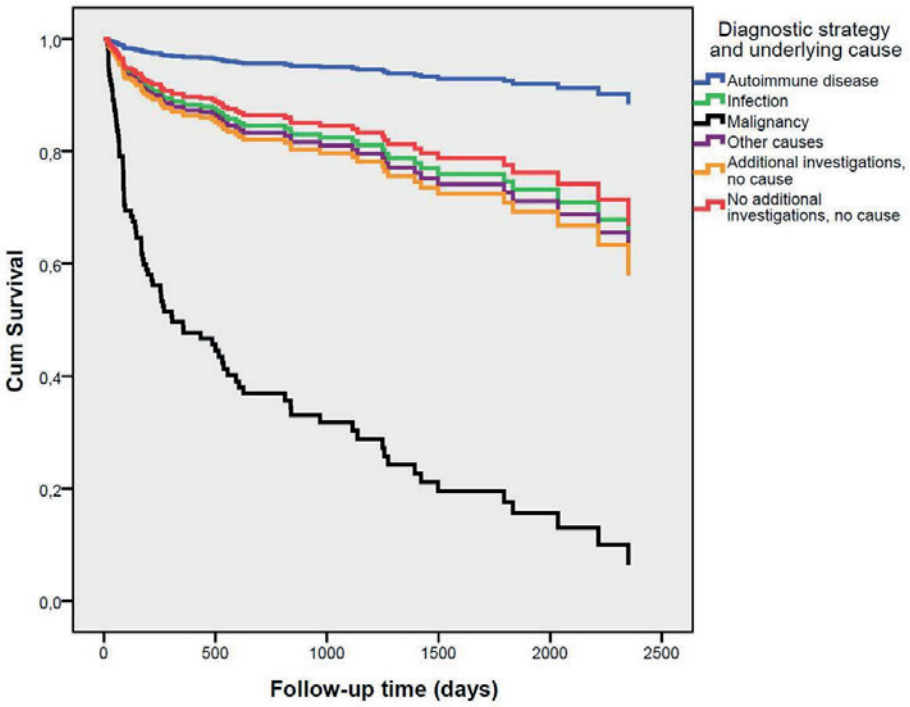


***Figure 1**, Survival analysis of ACD patients for the diagnostic strategy and underlying cause established. The survival of all ACD patients is plotted in different subpopulations. These subpopulations consist of the four main underlying causes of ACD, i.e. autoimmune disease (blue line), infection (green line), malignancy (black line) or other underlying causes (purple line). In addition, ACD patients, in whom no underlying cause could be established, are divided in additional investigations (orange line) and no additional investigations (red line).*

Discussion

Although many studies have investigated the pathophysiology of ACD and underlying causes in hospitalised patients, there is currently little knowledge regarding patients with newly diagnosed ACD in primary care. In this study, we described the diagnostic and therapeutic strategies of GPs in patients with newly diagnosed ACD and the underlying causes of ACD in community-dwelling adults.

Additional investigations were regularly performed by GPs. In 77% of patients, additional investigations were performed, while in 11%, the underlying cause of ACD was already known. Only 12% of patients did not undergo additional investigations, which are recommended in the national guideline. This may be due to the fact that these patients may have been considered too fragile for intensive diagnostics, due to low performance status, high age or co-morbidities. Therefore, GPs may consider additional investigations in view of a risk-benefit balance[12]. It is also possible that the underlying cause was not documented by the GP. Unfortunately, there was great variation in the completeness of the GPs' notes. GPs rarely record information concerning their instinct, which may affect diagnostic strategy.

If additional investigations were performed, the most common strategy was referral to a medical specialist (44%). This may be due to the diagnosis and treatment of the underlying cause being outside the scope of the GPs' focus. The severity of anaemia may be a plausible reason in many patients for referral, often leading to blood transfusion. In this study, a trend towards a lower haemoglobin was observed in patients referred to a medical specialist compared to patients who were not referred ($P = 0.064$).

The most common underlying causes of ACD in community-dwelling patients were infections (33%), autoimmune inflammation (23%) and malignancies (21%). These percentages are in agreement with data acquired in hospitalised ACD patients[13,14]. However, the prevalence of renal insufficiency among hospitalised ACD patients is much higher than the prevalence in our study population[13,14]. Patients who are admitted to hospital with severe illnesses have a higher rate of renal insufficiency. Patients with renal insufficiency may be more frequently hospitalised or are already being treated by a nephrologist, which leads to a lower incidence of renal insufficiency as a cause of newly diagnosed ACD in primary care.

When considering the survival of patients in whom an underlying causes could be established, marked differences were seen between the four groups. Those diagnosed with a malignancy exhibited the poorest survival. Remarkably, the survival plot did not show significant differences between the survival of patients with and without an established cause. It is possible that our study population was not large enough to show any effect.

The diagnostic and therapeutic strategy of GPs was in accordance with the national guideline, which recommends additional investigations unless the underlying causes is known, in combination with no oral iron supplementation, for 73% of ACD patients. The national guideline was more often applied, if patients presented with markedly increased ferritin levels. Since increased ferritin in a diagnostic parameter for ACD, noticeable elevated levels of ferritin might be an alarm symptom for GPs, which causes them to perform additional examinations more frequently. In addition, increased ferritin might exclude iron deficiency in the opinion of GPs, preventing the prescription of oral iron supplements.

Patients presenting with atypical complaints were also more often treated according to the national guideline. This might be due to the fact that atypical complaints convince GPs to order additional investigations or referral instead of the apparent clarity in the cases of patients with typical complains, leading to a decision against any investigations or referral.

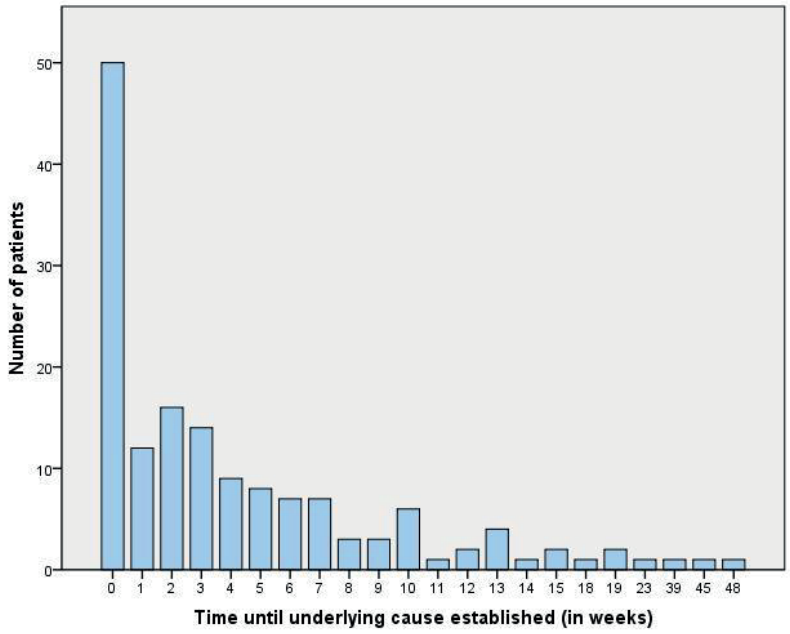
Although the adherence to the national guideline is high in this study, it appears that GPs prescribe oral iron supplementation in 13% of patients despite ferritin levels above 100 µg/L. Both lower haemoglobin and moderately raised ferritin levels are associated with an increased probability of iron description by the GP. Possibly, GPs are more likely to first treat anaemia with oral iron supplementation before determining the underlying causes of anaemia. The association of moderately raised ferritin levels and iron supplementation might be caused by the absence of a definite ferritin cut-off value defining iron deficiency.

In conclusion, although inappropriate oral iron supplementation is regularly prescribed, we demonstrated a high adherence to the national DCGP guideline. This study provides unique information on the diagnostic and therapeutic strategies of GPs' management of patients with newly diagnosed ACD.

References

1. Lipschitz D. Medical and functional consequences of anemia in the elderly. *J Am Geriatr Soc*. 2003;51(3 Suppl):S10-3.
2. Patel KV. Epidemiology of anemia in older adults. *Semin Hematol*. 2008;45(4):210-217.
3. Means RT,Jr. Recent developments in the anemia of chronic disease. *Curr Hematol Rep*. 2003;2(2):116-121.
4. Similowski T, Agusti A, MacNee W, Schonhofer B. The potential impact of anaemia of chronic disease in COPD. *Eur Respir J*. 2006;27(2):390-396.
5. Kollert F, Muller C, Tippelt A, et al. Anaemia in chronic respiratory failure. *Int J Clin Pract*. 2011;65(4):479-486.
6. Thomas MC, MacIsaac RJ, Tsalamandris C, Power D, Jerums G. Unrecognized anemia in patients with diabetes: A cross-sectional survey. *Diabetes Care*. 2003;26(4):1164-1169.
7. Ezekowitz JA, McAlister FA, Armstrong PW. Anemia is common in heart failure and is associated with poor outcomes: Insights from a cohort of 12 065 patients with new-onset heart failure. *Circulation*. 2003;107(2):223-225.
8. Kroot JJ, Tjalsma H, Fleming RE, Swinkels DW. Hepcidin in human iron disorders: Diagnostic implications. *Clin Chem*. 2011;57(12):1650-1669.
9. Shavelle RM, MacKenzie R, Paculdo DR. Anemia and mortality in older persons: Does the type of anemia affect survival? *Int J Hematol*. 2012;95(3):248-256.
10. Van Wijk MAM, Mel M, Muller PA, Zilverentand WGJ, Pijnenborg L, Kolnaar BGM. NHG-standaard anemie. *Huisarts & Wetenschap*. 2003;46:21-29.
11. Geïntegreerd klinisch chemisch laboratorium, laboratoriumgids versie 2011. http://www.resultlaboratorium.nl/professionals/.
12. Andres E, Serraj K, Federici L, Vogel T, Kaltenbach G. Anemia in elderly patients: New insight into an old disorder. *Geriatr Gerontol Int*. 2013;13(3):519-527.
13. Cash JM, Sears DA. The anemia of chronic disease: Spectrum of associated diseases in a series of unselected hospitalized patients. *Am J Med*. 1989;87(6):638-644.
14. Weiss G, Goodnough LT. Anemia of chronic disease. *N Engl J Med*. 2005;352(10):1011-1023.

Supplemental data



***Supplemental data 1**, Time in weeks until the underlying cause of ACD has been established.*

	Established	**Known**	**Established**		
	Metastatic		Local	Unknown metastatic	Total frequency
Lung	5		2	3	10
Prostate	2				2
Mamma	1	1			2
B-cell lymphoma	4		4		8
Colon				8	8
Gastric	2				2
Oesophagus	2				2
Elsewhere *	2		4	3	9
Unknown	6		1		7
Total	24	1	11	14	50

***Supplemental data 2**, Specification of malignancies established as underlying cause of the ACD. The primary location and metastatic stage of malignancies are shown. In addition, the malignancies are subdivided in either established after the patient was diagnosed with ACD or present at time of diagnosing ACD (designated as known). *Elsewhere comprised of single malignancies of the brain, larynx, penis, renal, hepatic, melanoma, pancreas, ovary and cervix.*

6

The impact of treatment strategy on mortality in iron deficiency anaemia

A. Schop
K. Stouten
J. van Rosmalen
J. Droogendijk
R.J. van Houten
J.A. Riedl
M-D. Levin

Submitted for publication

Abstract

Background: The current recommendations for the treatment of iron deficiency anaemia (IDA) in general practice consist of endoscopic evaluation and oral iron supplementation. In spite of the high effectiveness of endoscopic evaluation and the high prevalence of gastrointestinal malignancies in IDA patients, the rate of endoscopic evaluation by general practitioners remains low.

Methods: IDA was defined as haemoglobin below 13.7 g/dL (male) or 12.1 g/dL (female) and ferritin below 25 µg/L (male) or 20 µg/L (female). Information regarding IDA patients was obtained from the information systems of the participating GPs and the referral hospital. The gold standard for diagnosis and treatment of IDA was defined as a colonoscopy performed within four months after the discovery of IDA, combined with oral iron supplementation.

Results: A total of 242 patients with newly diagnosed IDA were analysed. Only 33% of these patients were treated according to the gold standard, which was mainly due to a low rate of colonoscopies (42%). Failure to adhere to the gold standard for treatment of IDA patients' age and gender were associated with an increased mortality (HR = 2.63 (95% CI 1.12-6.18), HR = 1.10 (95% CI 1.03-1.17) and HR = 2.05 (95% CI 1.01-4.13 for males) respectively). The gold standard was applied more frequently amongst male patients and those with increased transferrin levels (OR = 5.53 (95% CI 2.91-10.51) and OR = 2.33 (95% CI 1.18-4.62), respectively). In addition, the gold standard was less likely to be applied in patients with less decreased haemoglobin levels (OR = 0.79 per mmol/L increase in haemoglobin level (95% CI 0.66-0.95)).

Conclusions: In IDA patients diagnosed in general practice, a lack of adherence to the gold standard, as well as gender and age, were all associated with an increased mortality risk.

Introduction

Anaemia is a common finding in general practice with a prevalence of World Health Organisation (WHO)-defined anaemia of more than 10% in adults aged 65 years and older[1,2]. Up to a third of anaemia cases in general practice is related to nutrient deficiency, with a large proportion of cases due to iron deficiency anaemia (IDA)[3]. In the Western world, IDA originates mainly from diminished absorption of iron within the gastrointestinal tract (GI-tract) or iron loss due to chronic bleeding. The most common causes of chronic bleeding are menstrual bleeding, frequent blood donation, gastrointestinal blood loss due to polyps, inflammation and malignancies with active bleeding[4,5]. The current recommendations for the treatment of IDA in men and postmenopausal women consist of a colonoscopy (preferably in combination with a gastroscopy) and oral iron supplementation[4-6].

Previous studies showed that 31 - 47% of IDA patients in general practice receive an endoscopic evaluation[7-10]. In 23 - 86% of these evaluated cases, a lesion potentially responsible for GI blood loss is detected and in 2 - 17% of cases this turns out to be a GI malignancy[7-13]. In spite of the effectiveness of endoscopic evaluation and the high prevalence of GI-tract malignancies in IDA patients, the rate of endoscopic evaluation by general practitioners (GP) remains low in this patient group. This low adherence to endoscopic evaluation may have severe consequences for IDA patients. Therefore this study investigated the frequency with which the recommended treatment (i.e. a colonoscopy in combination with oral iron supplementation) was applied to IDA patients in general practice and whether treatment following the current recommendations influenced the survival of IDA patients. The factors associated with adherence to the recommendations were also investigated.

Methods

This study was approved by the ethics committee of the Albert Schweitzer Hospital and the participating GPs consented to the use of anonymous medical information.

Patient inclusion

From the 1st of February 2007 to the 1st of February 2013, all patients aged 50 years and older and presenting to their participating GPs with newly diagnosed IDA (i.e. no anaemia in the previous two years) were included in the study. Anaemia was defined as haemoglobin below 13.7 g/dL (8.5 mmol/L) for males and below 12.1 g/dL (7.5 mmol/L) for females according to the Dutch general practitioners guideline[6]. Iron deficiency was defined as ferritin below 25 µg/l for males and below 20 µg/l for females (i.e. lower limit of normal range)[14]. In order to ensure consistency amongst the laboratory analyses and

the criteria used to define IDA, all analyses were performed in the Clinical Chemistry laboratory of the Albert Schweitzer Hospital.

Data collection

We defined the current recommendations for the treatment of newly discovered IDA in general practice as the gold standard, consisting of (I) a colonoscopy (with or without a gastroscopy) performed within 4 months after discovery of IDA and (II) oral iron supplementation. The four month interval for performing a colonoscopy was based on the study of Ioannou *et al*[15]. All data was retrospectively collected from the information systems of both the GPs and the referral hospital. Colonoscopies requested by GPs and prescription of oral iron supplementation were recorded. In addition, predictors of survival (i.e. age, gender, and haemoglobin, ferritin and transferrin levels), and date of death or end of follow-up were collected. The end of follow-up was defined as the date of data collection from the information system or date of death.

Statistical analysis

The data was analysed using Statistical Package for Social Sciences (SPSS) for Windows, version 18. The patient population was characterized by standard descriptive statistics, using frequency counts and percentages for categorical variables, and medians and interquartile ranges for continuous variables. The patient population was divided into two groups, based on whether the gold standard treatment had or had not been applied. Kaplan-Meier curves were used to describe the mortality within each group. Differences between groups were analysed with the Fisher's exact test for categorical variables, the log-rank test for mortality, and the Mann-Whitney U test for continuous variables.

Multivariable logistic regression analysis was used to assess whether age, gender, haemoglobin, ferritin, and transferrin were associated with the probability of receiving the gold standard treatment. Cox proportional hazards analysis was used to estimate the association between receiving treatment according to the gold standard and mortality, with adjustment for all the covariates included in the logistic regression analysis. The proportional hazards assumption was assessed by testing the interaction effects of the covariates with time in a time-dependent Cox proportional hazards analysis. Because the proportional hazards assumption was violated for age, we stratified the Cox proportional hazards analysis by age categories, using four categories based on the quartiles of the observed age distribution. With this approach, the baseline hazard function is allowed to differ between age categories. To correct for residual confounding, age was still included

	Gold standard applied	Gold standard not applied	Total	P-value
	(N = 80) N (%)	(N = 162) N (%)	(N = 242) N (%)	
Gender				<0.001[a]
- Male	48 (60.0)	46 (28.4)	94 (38.8)	
- Female	32 (40.0)	116 (71.6)	148 (61.2)	
Deceased	8 (10.0)	37 (22.8)	45 (18.6)	0.018[b]
- Male	6 (7.5)	11 (6.8)	17 (7.0)	
- Female	2 (2.5)	26 (16.0)	28 (11.6)	
	Median (IQR)	Median (IQR)	Median (IQR)	
Age (years)	64 (58-77)	66 (52-80)	66 (54-79)	0.935[c]
- Male	64 (58-77)	66 (61-72)	66 (59-74)	
- Female	64 (54-79)	64 (52-81)	64 (52-81)	
Follow-up (months)	34 (21-63)	36 (19-54)	34 (20-56)	0.462[c]
Haemoglobin (g/dl)				0.264[c]
- Male (r: 13.7-18.0)	11.6 (9.1-13.0)	12.5 (10.8-13.1)	12.2 (9.9-13.0)	
- Female (r: 12.1-15.1)	9.4 (7.5-11.6)	11.1 (9.7-11.3)	10.9 (9.2-11.6)	
Ferritin (µg/l)				0.322[c]
- Male (r: 25-250)	10 (5-13)	9 (7-13)	10 (6-13)	
- Female (r:20-250)	6 (4-10)	8 (5-12)	8 (4-12)	
Transferrin (r: 6.0-8.0 g/dl)	3.6 (3.2-3.8)	3.3 (2.9-3.6)	3.4 (3.1-3.7)	0.002[c]

Table 1, *Characteristics of IDA patients. The number, percentages, median and interquartile ranges of the basic characteristics of both cohorts (i.e. gold standard applied or not) and the total population are shown. All laboratory tests were conducted in the Clinical Chemistry laboratory of the Albert Schweitzer Hospital. Abbreviations: r = reference value. Statistical tests used: a) Fisher's exact test, b) log rank test and c) Mann-Whitney U test.*

as a continuous covariate in the Cox proportional hazards analysis. Two-sided P-values lower than 0.05 were considered statistically significant.

Results

Patient characteristics

A total of 317 patients presenting with IDA were identified. No records were available for 75 patients, leaving a total of 242 patients (76%) with newly discovered IDA for analysis. A total of 80 patients (33%) were treated according to the gold standard, while 162 patients (67%) were not. Basic characteristics of both groups can be found in Table 1.

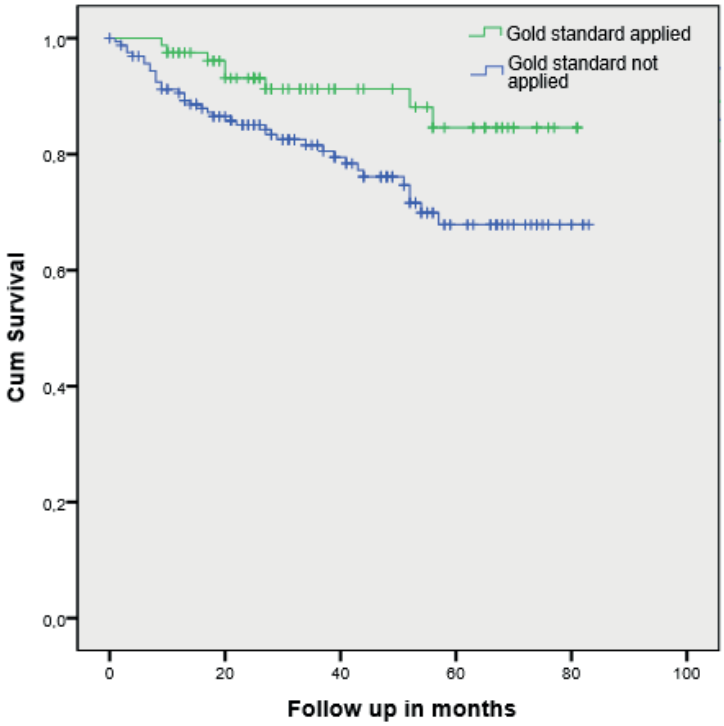


Figure 1, *Kaplan-Meier of IDA patients stratified by the use of the gold standard**.** Kaplan-Meier estimates of the survival of IDA patients in whom the gold standard is applied (green line) and in whom the gold standard is not applied (blue line).*

	Hazard ratio (95% CI)	P-value
Age (years)	1.10 (1.03-1.17)	0.007
Gender	2.05 (1.01-4.13)	0.046
Gold standard	2.63 (1.12-6.18)	0.03
Haemoglobin (g/dl)	0.90 (0.77-1.04)	0.16
Ferritin (µg/l)	1.01 (0.95-1.09)	0.72
Transferrin (g/dl)	0.88 (0.47-1.65)	0.70

Table 2, *multivariate analysis of the survival of IDA patients. Multivariable Cox proportional hazards analysis using gender (male versus female), gold standard (not applied versus applied) and age, haemoglobin, ferritin and transferrin as continuous variables.*

Survival analysis

Of the 80 patients for whom the gold standard was applied, 8 (10%) died during the follow-up period. Of the 162 patients, who had not been treated according to the gold standard, 37 (23%) died during the study period. The Kaplan-Meier estimates of survival of

both groups are shown in Figure 1. The log rank test showed a P-value of 0.018.
After stratification by age category, the test of the proportional hazard assumption in the Cox regression showed non-significant P-values for the interaction of time with age (P = 0.43), gender (P = 0.58), application of the gold standard (P = 0.50), haemoglobin (P = 0.24), ferritin (P = 0.23) and transferrin (P = 0.28), thereby confirming the validity of the proportional hazard assumption in this model. The Cox proportional hazards analysis indicated that male gender was significantly associated with increased mortality risk (HR=2.05, 95% CI 1.01-4.13, P = 0.046). In addition, failure to apply the gold standard in IDA patients was associated with increased mortality (HR = 2.63, 95% CI 1.12-6.18, P = 0.03).The hazard ratios for all covariates can be found in Table 2.

	Male N (%)	**Female N (%)**	**Total N (%)**
Colonoscopy	9 (10)	12 (8)	21 (9)
Iron supplementation	23 (24)	87 (59)	110 (45)
Gold standard (i.e. colonoscopy and iron supplementation)	48 (51)	32 (22)	80 (33)
No colonoscopy and no iron supplementation	14 (15)	17 (11)	31 (13)
Total	94 (39)	148 (61)	242 (100)

Table 3, *Treatment strategy of general practitioners. Number of requested colonoscopies and iron supplementation are shown for male and female IDA patients separately. In addition, the number of times the gold standard (i.e. colonoscopy in combination with iron supplementation) was applied by GPs is shown.*

	Odds ratio (95% CI)	**P-value**
Age (years)	0.99 (0.96-1.01)	0.30
Gender (male)	5.53 (2.91-10.51)	<0.001
Haemoglobin (g/dl)	0.79 (0.66-0.95)	0.01
Ferritin (µg/l)	1.02 (0.95-1.09)	0.68
Transferrin (g/dl)	2.33 (1.18-4.62)	0.02

Table 4, *Factors associated with adherence to the gold standard. Multivariable logistic regression analysis using gender (male versus female) as categorical variable and age, haemoglobin, ferritin and transferrin as continuous variables.*

Adherence to the gold standard

In 101 of 242 patients (42%), a colonoscopy was requested by the GP after IDA was established. In addition, 190 patients (77%) received oral iron supplementation (Table 3). Male gender (OR = 5.53 (P < 0.001)) and increased transferrin levels (OR = 2.33 (P = 0.02)) were associated with a higher probability of the application of the gold standard (Table 4). In addition, the gold standard was less often applied in patients with less decreased haemoglobin levels (OR = 0.79 per mmol/L increase in haemoglobin level (95% CI 0.66-0.95))**.**

Discussion

Summary

For IDA patients in general practice, the gold standard of treatment has been defined as a colonoscopy combined with oral iron supplementation. Non-adherence to this gold standard was associated with an increased mortality risk in this study, as were age and male gender. Furthermore, the gold standard was applied in only 33% of IDA patients, mainly due to a low rate of colonoscopies. This study also showed that the gold standard was more often applied in males and patients with increased transferrin levels and less often applied in patients with less decreased haemoglobin levels.

Strengths and limitations

To the best of the authors' knowledge, this is the first study describing the effect of the treatment strategy of IDA patients on mortality in general practice. A possible limitation of this study setting is the great variation in the completeness of the GPs' notes. GPs rarely record information concerning their instinct and thought processes, which may affect the diagnostic strategy. They may be greatly influenced by the patients' age, co-morbidities or wishes regarding invasive diagnostics and treatment, factors that generally remained unrecorded.
Due to the retrospective design and incomplete notes of GPs, the survival analysis could not be corrected for any possible co-morbidities of the IDA patients. In spite of this limitation, we were able to correct our model for several other important factors, such as age and laboratory parameters detailing iron status, and still found a strong association between an increased mortality risk and non-adherence to the gold standard. Despite the drawbacks of our study, it was shown that treatment strategy can significantly influence mortality. Further research on the relationship between treatment strategy and mortality should be undertaken to further elucidate this association.

Comparison with existing literature

This study demonstrated that IDA patients who were not treated according to the gold standard have an increased mortality risk, as compared to patients to whom this standard was applied. There are two possible explanations for this association. First, the GP takes not only the laboratory measurements into consideration, but also the patient's overall health, age and wishes regarding invasive diagnostics. In this way, GPs may filter out those patients for whom the potential benefits of the invasive procedure of a colonoscopy and subsequent interventions no longer outweigh the risks. Especially in elderly patients, severe comorbidities and poor overall health status may prevent them from having a colonoscopy[10]. These 'high risk' patients may have caused the increased mortality risk in the untreated IDA patient. Although we were able to correct for age in the survival

analysis, we were unable to correct for comorbidities and health status of the patients. Secondly, adherence to the gold standard may aid the early discovery of a GI-tract malignancy[16,17]. This early discovery may lead to an improved cancer-related prognosis and thus to a decreased mortality for IDA patients who are treated according to the gold standard.
In addition, the survival analysis in this study demonstrated lower survival rate in male patients, which may be explained by a higher incidence of colon carcinoma in males or by the lower overall survival rate of men in the Netherlands[18,19]. Females are often found to have a benign underlying cause of IDA (i.e. hypermenorrhoea), while males have *a priori* more likely chance for a malignant cause.

Only 42% of newly discovered IDA patients received a colonoscopy in the present study, which is in line with existing literature[7-10]. The cause for this low percentage of IDA patients receiving a colonoscopy is unknown, since GPs rarely document the reason or reasons for refraining from performing one. In this study population, females were more likely to be excluded from a colonoscopy. No other common characteristic (e.g. advanced age) was found.
In the present study, the gold standard for treatment of IDA patients was more often applied to males. This effect has been observed before[7]. This may be due to the higher incidence of colon carcinoma in men in the Netherlands, which could alert the GP and lead to a higher adherence to the gold standard in case the patient is male[18]. In addition, the inclusion criteria of this study only allowed for females aged 50 years and older in order to exclude a predominance of hypermenorrhoea as the cause of IDA. However, this age limit does not completely exclude all menstruating females. Therefore, it is possible that fewer females were referred for a colonoscopy by GPs since hypermenorrhoea was still present and these patients did not require further diagnostics according to the current recommendations[6].
Finally, increased transferrin levels were associated with a greater adherence to the gold standard, while less decreased haemoglobin levels were associated with a lower probability of adherence to the gold standard. It is possible that GPs may consider the severity of the anaemia when deciding whether or not to apply the gold standard for treatment of IDA patients. Increased transferrin levels are observed more often in patients with severe anaemia (data not shown). In addition, a recent study showed that IDA patients with GI malignancies have significantly lower haemoglobin levels than IDA patients without GI malignancies[7]. These factors may also cause the GPs to adhere more strictly to the gold standard.

Implications for research and/or practice

The lack of adherence to the gold standard in treatment of IDA patients was found to be associated with an increased mortality risk in general practice. This may be due to the

detrimental effects of a late diagnosis of colon carcinoma or due to existing comorbidities. Although we were able to correct our analysis for age, further research is necessary to investigate the influence of other predictors, such as co-morbidities and overall health status, on the survival of IDA patients.

References

1. Patel KV. Epidemiology of anemia in older adults. *Semin Hematol*. 2008;45(4):210-217.
2. Guralnik JM, Eisenstaedt RS, Ferrucci L, Klein HG, Woodman RC. Prevalence of anemia in persons 65 years and older in the united states: Evidence for a high rate of unexplained anemia. *Blood*. 2004;104(8):2263-2268.
3. Kassebaum NJ, Jasrasaria R, Naghavi M, et al. A systematic analysis of global anemia burden from 1990 to 2010. *Blood*. 2014;123(5):615-624.
4. Polin V, Coriat R, Perkins G, et al. Iron deficiency: From diagnosis to treatment. *Dig Liver Dis*. 2013;45(10):803-809.
5. Clark SF. Iron deficiency anemia. *Nutr Clin Pract*. 2008;23(2):128-141.
6. Van Wijk MAM, Mel M, Muller PA, Zilverentand WGJ, Pijnenborg L, Kolnaar BGM. NHG-standaard anemie. *Huisarts & Wetenschap*. 2003;46:21-29.
7. Droogendijk J, Beukers R, Berendes PB, Tax MG, Sonneveld P, Levin MD. Screening for gastrointestinal malignancy in patients with iron deficiency anemia by general practitioners: An observational study. *Scand J Gastroenterol*. 2011;46(9):1105-1110.
8. Lucas CA, Logan EC, Logan RF. Audit of the investigation and outcome of iron-deficiency anaemia in one health district. *J R Coll Physicians Lond*. 1996;30(1):33-36.
9. Logan EC, Yates JM, Stewart RM, Fielding K, Kendrick D. Investigation and management of iron deficiency anaemia in general practice: A cluster randomised controlled trial of a simple management prompt. *Postgrad Med J*. 2002;78(923):533-537.
10. Yates JM, Logan EC, Stewart RM. Iron deficiency anaemia in general practice: Clinical outcomes over three years and factors influencing diagnostic investigations. *Postgrad Med J*. 2004;80(945):405-410.
11. McIntyre AS, Long RG. Prospective survey of investigations in outpatients referred with iron deficiency anaemia. *Gut*. 1993;34(8):1102-1107.
12. Cook IJ, Pavli P, Riley JW, Goulston KJ, Dent OF. Gastrointestinal investigation of iron deficiency anaemia. *Br Med J (Clin Res Ed)*. 1986;292(6532):1380-1382.
13. Rockey DC, Cello JP. Evaluation of the gastrointestinal tract in patients with iron-deficiency anemia. *N Engl J Med*. 1993;329(23):1691-1695.
14. Geïntegreerd klinisch chemisch laboratorium, laboratoriumgids versie 2011. http://www.resultlaboratorium.nl/professionals/.
15. Ioannou GN, Rockey DC, Bryson CL, Weiss NS. Iron deficiency and gastrointestinal malignancy: A population-based cohort study. *Am J Med*. 2002;113(4):276-280.
16. Levin B, Lieberman DA, McFarland B, et al. Screening and surveillance for the early detection of colorectal cancer and adenomatous polyps, 2008: A joint guideline from the american cancer society, the US multi-society task force on colorectal cancer, and the american college of radiology. *Gastroenterology*. 2008;134(5):1570-1595.
17. Teng CL, Yu JT, Chen YH, Lin CH, Hwang WL. Early colonoscopy confers survival benefits on colon cancer patients with pre-existing iron deficiency anemia: A nationwide population-based study. *PLoS One*. 2014;9(1):e86714.
18. Gommer AM. Dikke darmkanker: Incidentie, prevalentie en sterfte. http://www.nationaalkompas.nl. Accessed March, 2014.
19. Centraal Bureau voor Statistiek (CBS). Gezonde levensverwachting, vanaf 1981. http://statline.cbs.nl/StatWeb/publication/?VW=T&DM=SLNL&PA=71950NED&D1=0-2,5,7&D2=a&D3=a&D4=a&D5=l&HD=121001-1214&HDR=G4,G3,T&STB=G1,G2. Accessed November, 2015.

PART II

Digital microscopy and leukocyte morphology

7

Inter-laboratory reproducibility of blood morphology using the digital microscope

J.A. Riedl
K. Stouten
H. Ceelie
J. Boonstra
M-D. Levin
W. van Gelder

J. Lab Autom. 2015 Dec; 20(6): 670-675

Abstract

Differential counting of peripheral blood cells is an important diagnostic tool. However, manual morphological analysis using the microscope is time-consuming and requires highly trained personnel. The digital microscope is capable of performing an automated peripheral blood cell differential, which is as reliable as manual classification by experienced laboratory technicians. To date, information concerning the inter-laboratory variation and quality of cell classification by independently operated digital microscopy systems is limited. We compared four independently operated digital microscope systems for their ability in classifying the five main peripheral blood cell classes and detection of blast cells in 200 randomly selected samples. Set against the averaged results, the R^2 values for neutrophils ranged between 0.90 and 0.96, for lymphocytes between 0.83 and 0.94, for monocytes between 0.77 and 0.82, for eosinophils between 0.70 and 0.78, and for blast cells between 0.94 and 0.99. The R^2 values for the basophils were between 0.28 and 0.34. This study shows that independently operated digital microscope systems yield reproducible pre-classification results when determining the percentages of neutrophils, eosinophils, lymphocytes, monocytes, and blast cells in a peripheral blood smear. Detection of basophils was hampered by the low incidence of this cell class in the samples.

Introduction

Morphological analysis of blood cells is invaluable to patient management by the clinician. Until now, manual morphological assessment using the microscope has been set as the gold standard. However, manual assessment of a blood smear is subject to individual interpretation of images, resulting in significant inter-observer variability[1-3]. In addition, correct morphological classification is labour-intensive and requires continuous training of laboratory personnel. Automated digital morphological assessment of blood cells is therefore considered a valuable development, as it can overcome these drawbacks. The digital microscope (DM) offers several advantages. First, the DM ensures the constant presence of a morphological expert system in the routine laboratory. Second, the system stores an image of every analysed cell, thereby offering the ability to re-evaluate cell types with colleagues and other pathology experts, either directly or by using tele-haematology[3-6]. Finally, the system enables us to digitally archive blood smears and body fluid samples indefinitely.

Since the 1970s, several automated image processing devices have been developed by various manufacturers[7]. It was previously shown that a DM system, using several advanced mathematical algorithms, is capable of correct classification of leukocytes in peripheral blood and body fluid samples in relation to manual microscopic assessment of the five main peripheral blood cell categories[3-5,8-10]. An overall accuracy of 92.0% was found when the pre-classification results of the DM96 (Cellavision, Lund, Sweden) were compared to those of manual assessment[3,11]. It has been shown that the classification performance of this particular system is as reliable as manual classification by experienced laboratory technicians in classifying the five main peripheral blood cell categories[3].

Only limited information is currently available regarding inter-laboratory variation and quality in cell classification by independently operated digital microscope systems. As part of the continuing validation of DM systems, it is important to assess the inter-laboratory variation between systems operated at different locations and determine whether they can produce comparable results. To achieve this, we compared four independently operated DM systems when analysing randomly selected samples.

Materials and methods

Digital microscope systems and locations

In this study we set out to compare four independently operated digital microscope systems (DM96) for their ability to classify the five main peripheral blood cell classes (neutrophils, lymphocytes, monocytes, eosinophils, and basophils) and blast cells in 200 samples. The DM machines were located at four different clinical chemistry laboratories in the Netherlands: the Albert Schweitzer Hospital (ASz), the Vlietland Hospital (Vlietland),

the Erasmus Medical Centre, central location (Centrum), and the Erasmus Medical Centre Daniel den Hoed Cancer Clinic (Daniel).

Blood sample collection and analysis

Using standardised protocols, each laboratory collected peripheral blood samples from 50 randomly selected patients and generated four blood smear specimens per sample. Each hospital location received one smear specimen from each patient. A total of 200 specimens were analysed at each location. Prior to sample analysis, all four DM systems were calibrated using a calibration slide. In addition, each system was set to analyse and classify 200 leukocytes per sample. At each location the samples were processed on the DM by two local technicians following standardised procedures. This study focused on the pre-classification results obtained from the four different DM systems. Pre-classification is defined as the initial classification performed by the DM, without intervention or correction by the local operator. Therefore, results could not be influenced by manual interference.

Inter-laboratory variation

The number of each cell type found (neutrophils, lymphocytes, monocytes, eosinophils, basophils, and blast cells) was expressed as a percentage of the total amount of cells classified. For each location, the individual pre-classification result per cell class was compared to the averaged percentage of the other three locations in order to determine the inter-laboratory variation.

Statistics

The coefficient of determination (R^2) was calculated for each comparison in order to determine the inter-laboratory variation, using Statistical Package for the Social Sciences (SPSS) version 18 for Windows.

Results and Discussion

The total number of classified leukocytes did not reach 200 cells in all samples. The range in numbers and percentages per cell class per location is shown in Table 1. Figure 1-A-F shows scatter plots and the associated R^2 value for each comparison per cell class. Overall, small inter-laboratory variation was found for neutrophils (R^2 = 0.90-0.96), lymphocytes (R^2 = 0.83-0.94), monocytes (R^2 = 0.77-0.82), eosinophils (R^2 = 0.70-0.78), and blast cells (R^2 = 0.94-0.99). Only basophils showed a large variation (R^2 = 0.28-0.34).

	ASz		Centrum		Daniel		Vlietland	
	#	%	#	%	#	%	#	%
Neutrophils	2-189	1.1-96.9	0-192	0.0-97.0	0-191	0.0-97.4	0-190	0.0-96.9
Lymphocytes	0-184	0.0-92.4	1-179	0.5-92.7	2-187	1.0-93.5	1-178	0.5-90.8
Monocytes	1-73	0.5-38.2	1-65	0.5-35.7	0-75	0.0-39.3	0-75	0.0-40.8
Eosinophils	0-32	0.0-16.0	0-26	0.0-20.7	0-40	0.0-21.3	0-56	0.0-28.4
Basophils	0-12	0.0-7.7	0-11	0.0-6.0	0-10	0.0-6.5	0-10	0.0-5.4
Blast cells	0-151	0.0-82.1	0-137	0.0-86.2	0-167	0.0-88.4	0-123	0.0-73.7

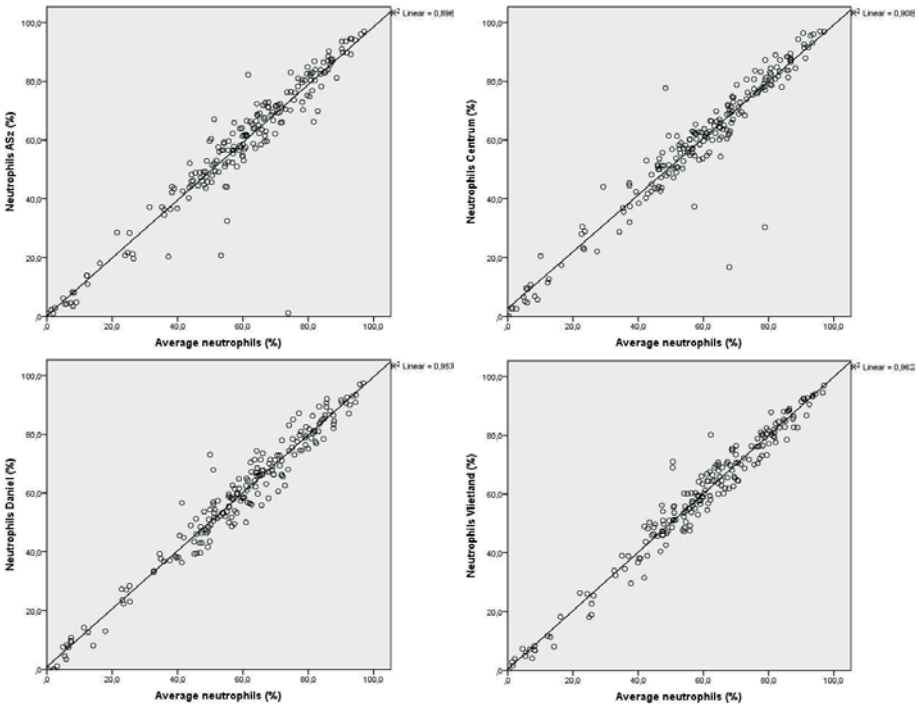
Table 1, *Ranges for each cell class found at the different locations (ASz, Centrum, Daniel, and Vlietland)*

As part of the continuing validation of DM systems for morphological analysis of a peripheral blood smear, the inter-laboratory variation for the five main blood cell classes and blast cells was determined. This is the first published study that considers this variation between independently operated digital microscopy systems. The pre-classification results show small inter-laboratory variation for four of the five main peripheral blood cell classes. This is comparable to R^2 values found when comparing two manual differential counts, as done by Ceelie *et al*[3]. The DM showed even less variation between several machines than between the two manual differentials for neutrophils (R^2 = 0.90 for manual count) and monocytes (R^2 = 0.65 for manual count)[3].

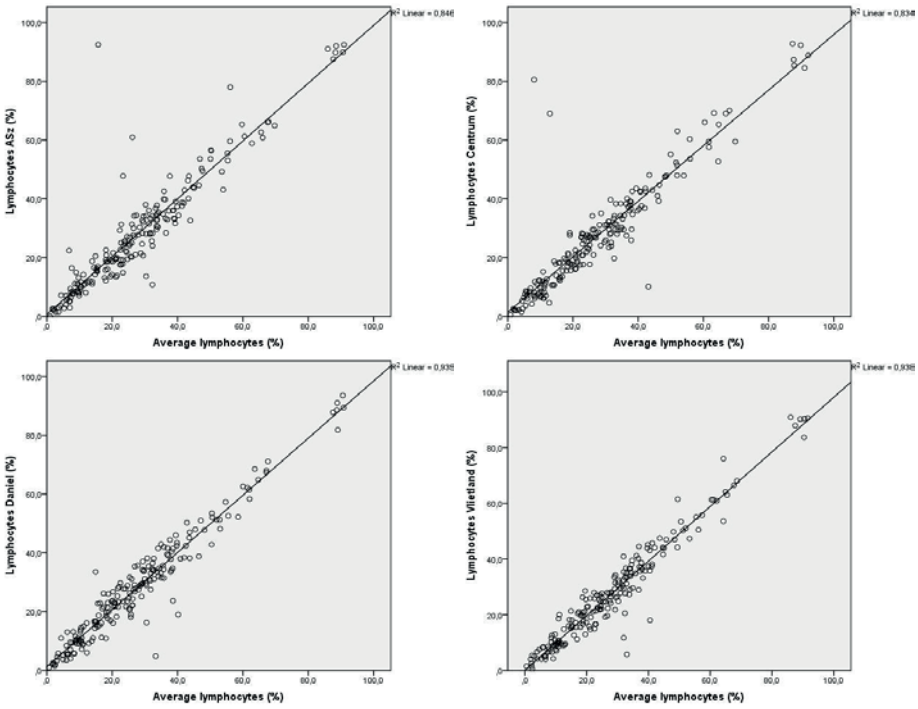
Blast cells were detected with an excellent accuracy, despite the fact that the overall average percentage of blast cells was low. This is probably due to the large spread in counted cells per sample. Not every sample contained blast cells, which lowers the overall average percentage. The same was observed when two manual counts of blast cells were compared, resulting in an R^2 value of 0.97[3]. Again, the DM showed even less variation between systems than between experienced morphologists, since R^2 values between 0.94 and 0.99 were found in this study.

Only the pre-classification results of the basophils showed considerable inter-laboratory variation. This variation was also seen when comparing manual assessment by an experienced morphologist to a reference differential, as done by Briggs *et al*[4]. Even an experienced morphologist could not achieve an R^2 value higher than 0.30 when manually classifying basophils. Briggs *et al*[4] also compared the manual differentials executed by two experienced morphologists with the DM. This resulted in an R^2 value of 0.00[4]. Similar results were found by Ceelie *et al*[3]when comparing two manual differential counts with each other and with the DM. The poor R^2 values for basophils are due to the low number of detected cells of this class per peripheral blood smear, leading to profound relative differences in detected percentages of basophils at different locations.

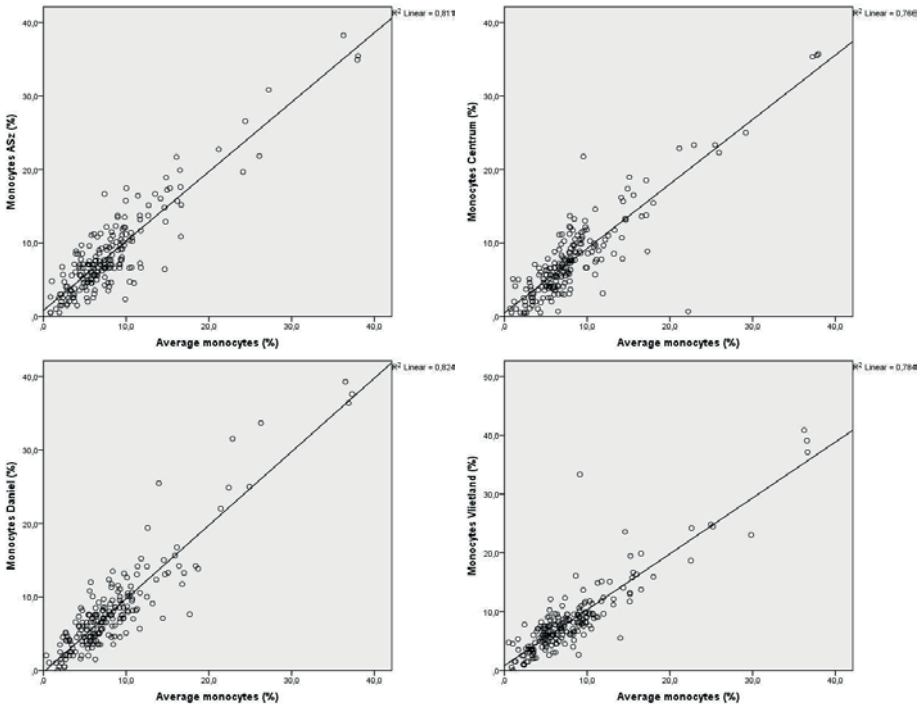
A


Neutrophils ASz (%)
Average neutrophils (%)
Neutrophils Centrum (%)
Average neutrophils (%)
Neutrophils Daniel (%)
Average neutrophils (%)
Neutrophils Vlietland (%)
Average neutrophils (%)

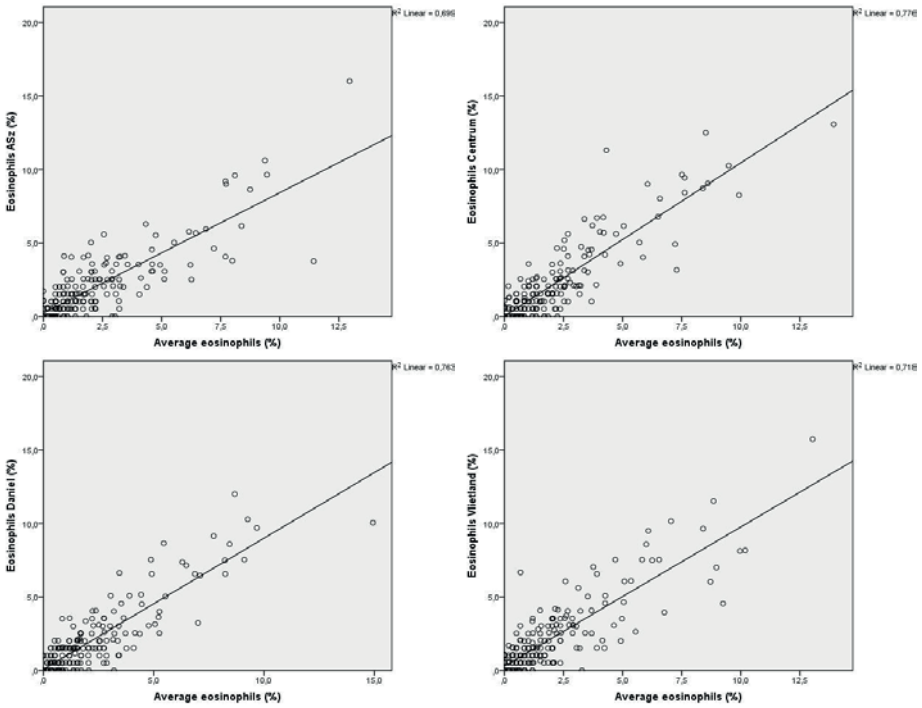
B


Lymphocytes ASz (%)
Average lymphocytes (%)
Lymphocytes Centrum (%)
Average lymphocytes (%)
Lymphocytes Daniel (%)
Average lymphocytes (%)
Lymphocytes Vlietland (%)
Average lymphocytes (%)

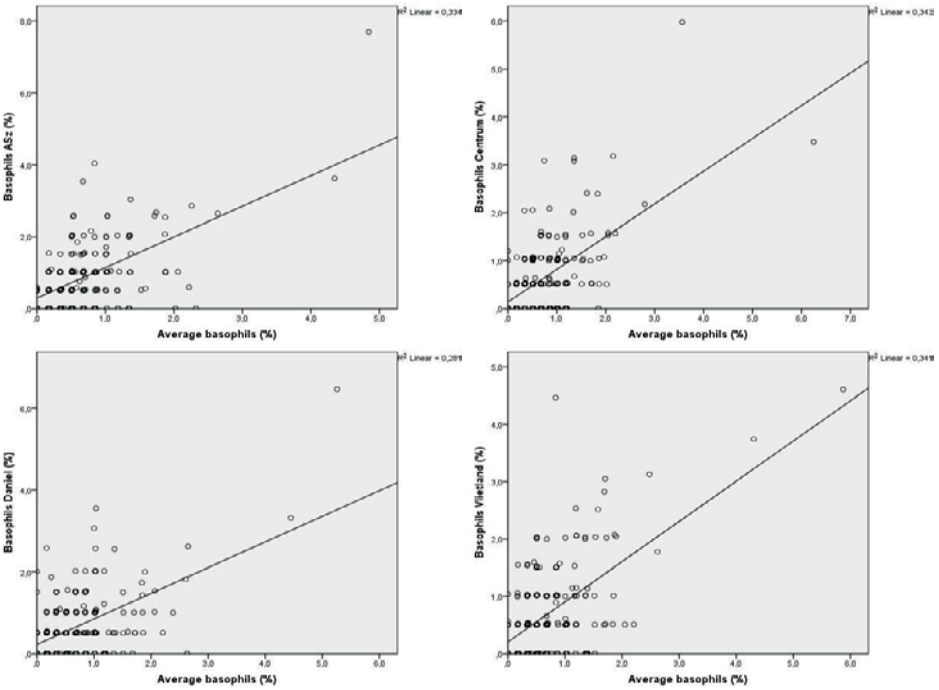
C


Monocytes ASz (%)
Average monocytes (%)
Monocytes Centrum (%)
Average monocytes (%)
Monocytes Daniel (%)
Average monocytes (%)
Monocytes Vlietland (%)
Average monocytes (%)

D


Eosinophils ASz (%)
Average eosinophils (%)
Eosinophils Centrum (%)
Average eosinophils (%)
Eosinophils Daniel (%)
Average eosinophils (%)
Eosinophils Vlietland (%)
Average eosinophils (%)

E


Basophils ASz (%)
Average basophils (%)
Basophils Centrum (%)
Average basophils (%)
Basophils Daniel (%)
Average basophils (%)
Basophils Vlietland (%)
Average basophils (%)

F

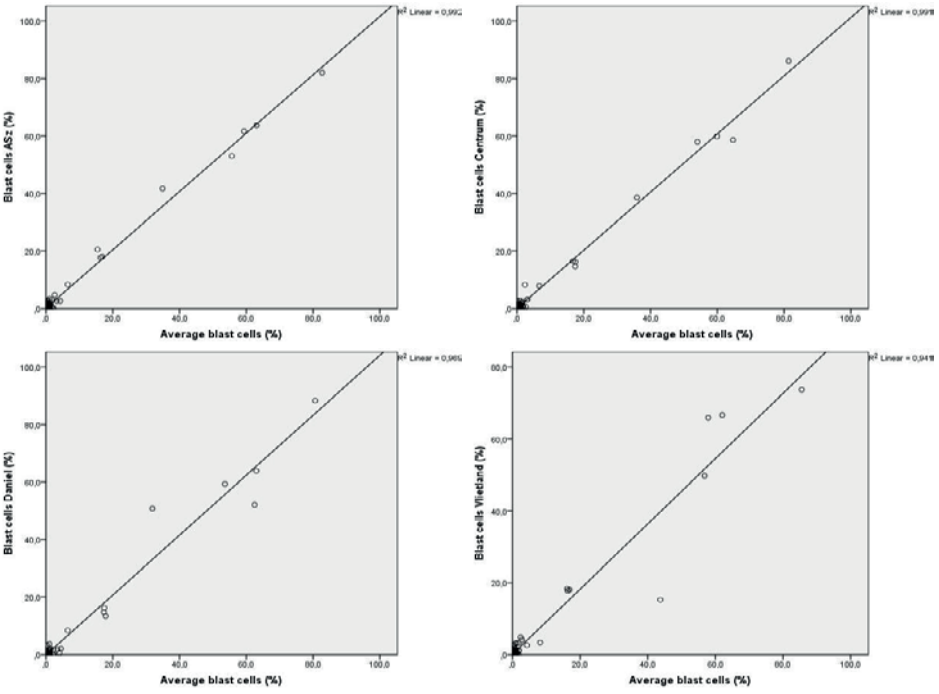

Blast cells ASz (%)
Average blast cells (%)
Blast cells Centrum (%)
Average blast cells (%)
Blast cells Daniel (%)
Average blast cells (%)
Blast cells Vlietland (%)
Average blast cells (%)

Figure 1 *(previous pages)**, Results of pre-classification comparisons for segmented neutrophils (A), lymphocytes (B), monocytes (C), eosinophils (D), basophils (E), and blast cells (F). The Y axis shows the percentages of classes found in each of the 200 samples per location (ASz, Centrum, Daniel, and Vlietland). The X axis shows the average percentage of the different cells classes found at the four locations excluding the location on the Y-axis.*

A database containing approximately 1.4 million leukocytes was set up to compare the pre-classification performance of the DM with the manual assessment of peripheral blood smears by experienced morphologists. This database yielded an R^2 value of 0.88 for the basophils (Riedl, data not yet published). The size of that database overcomes the problem encountered in this study, which was hampered by the low number of counted basophils in the various samples. The same was observed in a study by Lee *et al*[12], who did not include normal blood smears and therefore may have had more basophils than is usually observed. Their comparison between the DM96 and a manual count gave an R^2 value of 0.76[12].

In conclusion, this study shows that independently operated digital microscope systems, stationed at four different locations, yield reproducible pre-classification results when determining percentages of neutrophils, lymphocytes, monocytes, and eosinophils present in a blood smear. In addition, blast cells were also detected correctly and with only minor variation in detected percentages between the different microscopy systems. The classification of basophils was less accurate because of the low number of basophils present in these samples.

References

1. Rumke CL. Imprecision of ratio-derived differential leukocyte counts. *Blood Cells*. 1985;11(2):311-4, 315.
2. Bentley SA. Automated differential white cell counts: A critical appraisal. *Baillieres Clin Haematol*. 1990;3(4):851-869.
3. Ceelie H, Dinkelaar RB, van Gelder W. Examination of peripheral blood films using automated microscopy; evaluation of diffmaster octavia and cellavision DM96. *J Clin Pathol*. 2007;60(1):72-79.
4. Briggs C, Longair I, Slavik M, et al. Can automated blood film analysis replace the manual differential? an evaluation of the CellaVision DM96 automated image analysis system. *Int J Lab Hematol*. 2009;31(1):48-60.
5. Kratz A, Bengtsson HI, Casey JE, et al. Performance evaluation of the CellaVision DM96 system: WBC differentials by automated digital image analysis supported by an artificial neural network. *Am J Clin Pathol*. 2005;124(5):770-781.
6. Billard M, Lainey E, Armoogum P, Alberti C, Fenneteau O, Da Costa L. Evaluation of the CellaVision DM automated microscope in pediatrics. *Int J Lab Hematol*. 2010;32(5):530-538.
7. Tatsumi N, Pierre RV. Automated image processing. past, present, and future of blood cell morphology identification. *Clin Lab Med*. 2002;22(1):299-315, viii.
8. Riedl JA, Dinkelaar RB, van Gelder W. Automated morphological analysis of cells in body fluids by the digital microscopy system DM96. *J Clin Pathol*. 2010;63(6):538-543.
9. Swolin B, Simonsson P, Backman S, Lofqvist I, Bredin I, Johnsson M. Differential counting of blood leukocytes using automated microscopy and a decision support system based on artificial neural networks--evaluation of DiffMaster octavia. *Clin Lab Haematol*. 2003;25(3):139-147.
10. Rollins-Raval MA, Raval JS, Contis L. Experience with CellaVision DM96 for peripheral blood differentials in a large multi-center academic hospital system. *J Pathol Inform*. 2012;3:29.
11. Seeto M, Ledesma A, Duff J. Evaluation of the CellaVision DM96 system: Familiarization study in a large laboratory setting. *International Journal of Laboratory Haematology*. 2008;30(Suppl. 1):77.
12. Lee LH, Mansoor A, Wood B, Nelson H, Higa D, Naugler C. Performance of CellaVision DM96 in leukocyte classification. *J Pathol Inform*. 2013;4:14-3539.114205. Print 2013.

8

Examination of peripheral blood smears: performance evaluation of a digital microscopy system using a large-scale leukocyte database

K. Stouten
J.A. Riedl
M-D. Levin
W. van Gelder

Int J Lab Hematol. 2015 Oct; 37(5): e137-140

The analysis of blood morphology is of great diagnostic importance to the clinician. Manual morphological assessment using the microscope has been considered the gold standard for years but can be vulnerable to inter-observer variability, is labour intensive, and requires highly and continuously trained personnel[1-3]. An exciting development in the field is the introduction of digital microscope (DM) systems. A DM ensures the constant presence of a morphological expert in the routine laboratory and enables the automatic recognition of (pathological) cell types[3-5].

It was previously shown that the classification performance of the DM is equal to manual performance when classifying the five main peripheral blood cell classes (neutrophils, lymphocytes, monocytes, eosinophils, and basophils)[3-9]. However, these studies either used a low number of samples and cells or did not include a combination of normal and abnormal peripheral blood smears (PBS)[3-9]. Several studies also compared post-classification results (which include manual interference) with manual analysis preventing a clear view on the ability of the DM to correctly classify cells without manual interference[4,6,7]. Here, we present a large-scale database of about 1.4 million leukocytes from both normal and abnormal PBS, pitting the DM's pre-classification performance against the gold standard.

Methods

Patient samples

Venous blood was collected using K3-EDTA as anticoagulant and stored at room temperature until further analysis. Within 4 hours of collection, blood smears were prepared and stained according to Romanowsky (May-Grunwald/Giemsa/Wright), using an automated slide preparation unit (SP-100, Sysmex, Kobe, Japan). The number of cells analysed per slide was set at 200, both for manual assessment and assessment by the DM. Manual assessment, set as the gold standard, was defined as analysis of a slide by an experienced morphology expert by reviewing the digital images provided by the DM. The pre-classification performance, defined as the initial classification by the DM without manual intervention, was compared to this gold standard. The samples were selected from our laboratory which handles routine samples from both general practitioners and hospitals, including a haemato-oncology ward.

Automated microscopy system

For this study, the DM96 (Cellavision, Lund, Sweden), described in reference 3, was used as DM system, operated with the Cellavision Blood differential module (Version 2.0).

Statistics

Statistical analysis was carried out using Statistical Package for Social Sciences (SPSS) version 18 and Medcalc version 15, both for Windows. The study design was based on the National Committee for Clinical Laboratory Standard document H20A. To determine accuracy, the percentage of each class per sample found by the DM was compared to the percentage found by the manual assessment. Results were analysed according to Bland and Altman[10], and by determining the Pearson product-moment correlation coefficient (R). To generate Bland-Altman plots, the difference (percentage DM minus percentage expert) per sample was shown as a function of the mean result of both DM and expert. Mean difference and limits of agreement (mean difference ± 1.96*standard deviation) were determined and added to the plots as reference lines. Constant and proportional biases were assessed using Deming regression.

Results

A total of 6945 PBS were analysed with approximately 1.4 million classified cells. When possible, 200 cells per sample were analysed. In 214 cases (3.1%), the DM did not reach the number of 200 cells due to leukopenic samples. In 57 cases (0.8%), the number of counted cells was below 100 (minimum 16 cells). As the number of cells in each class was expressed as a percentage of the total number of counted cells, these samples were included in the analysis.

The results for the different leukocyte classes and nucleated red blood cells (NRBCs) can be found in Table 1 and Figure 1. Pre-classification performance has an excellent accuracy for the five main blood cell classes and NRBCs. The DM software is currently unable to recognise promonocytes, prolymphocytes, hairy cells, and cleaved cells; these were only counted by the experts.

Proportional bias was found to be present for the five main classes and metamyelocytes and ranged from 0.5% for neutrophils to 23.3% for metamyelocytes. Constant bias was found to be present for nine classes and ranged from 0.04% for basophils to 0.39% for blast cells.

For blast cells, the limit for a positive finding was set at 0 for the DM, defining a percentage above 0.0% as positive. With this limit, the DM achieved a blast cell sensitivity of 100% with a specificity of 67%.

Cell classes	Correlation	Mean difference	Limits of agreement	Regression equation	95% CI intercept	95% CI slope
Neutrophils	0.997	-0.147	-3.385-3.091	0.16+0.99x	0.05-0.27*	0.99-0.99*
Lymphocytes	0.995	-0.461	-4.767-3.845	0.38+0.97x	0.31-0.46*	0.97-0.98*
Monocytes	0.933	-0.151	-4.140-3.838	0.21+0.95x	-0.10-0.51	0.90-0.99*
Eosinophils	0.978	-0.099	-1.339-1.141	0.09+0.91x	0.07-0.11*	0.90-0.92*
Basophils	0.928	-0.062	-0.864-0.740	0.04+0.84x	0.01-0.07*	0.80-0.89*
Blast cells	0.840	0.367	-3.298-4.032	0.39+0.93x	0.36-0.41*	0.79-1.07
Promyelocytes	0.432	0.027	-1.361-1.415	0.05+0.60x	-0.01-0.10	-0.99-2.18
Myelocytes	0.808	0.139	-1.707-1.985	0.16+0.94x	0.08-0.25*	0.68-1.21
Metamyelocytes	0.802	-0.021	-2.037-1.995	0.09+0.77x	0.02-0.15*	0.59-0.95*
Plasma cells	0.576	0.114	-1.121-1.349	0.13+0.67x	0.09-0.16*	-0.61-1.94
NRBCs	0.958	0.333	-1.660-2.326	0.32+1.03x	0.30-0.34*	0.99-1.06

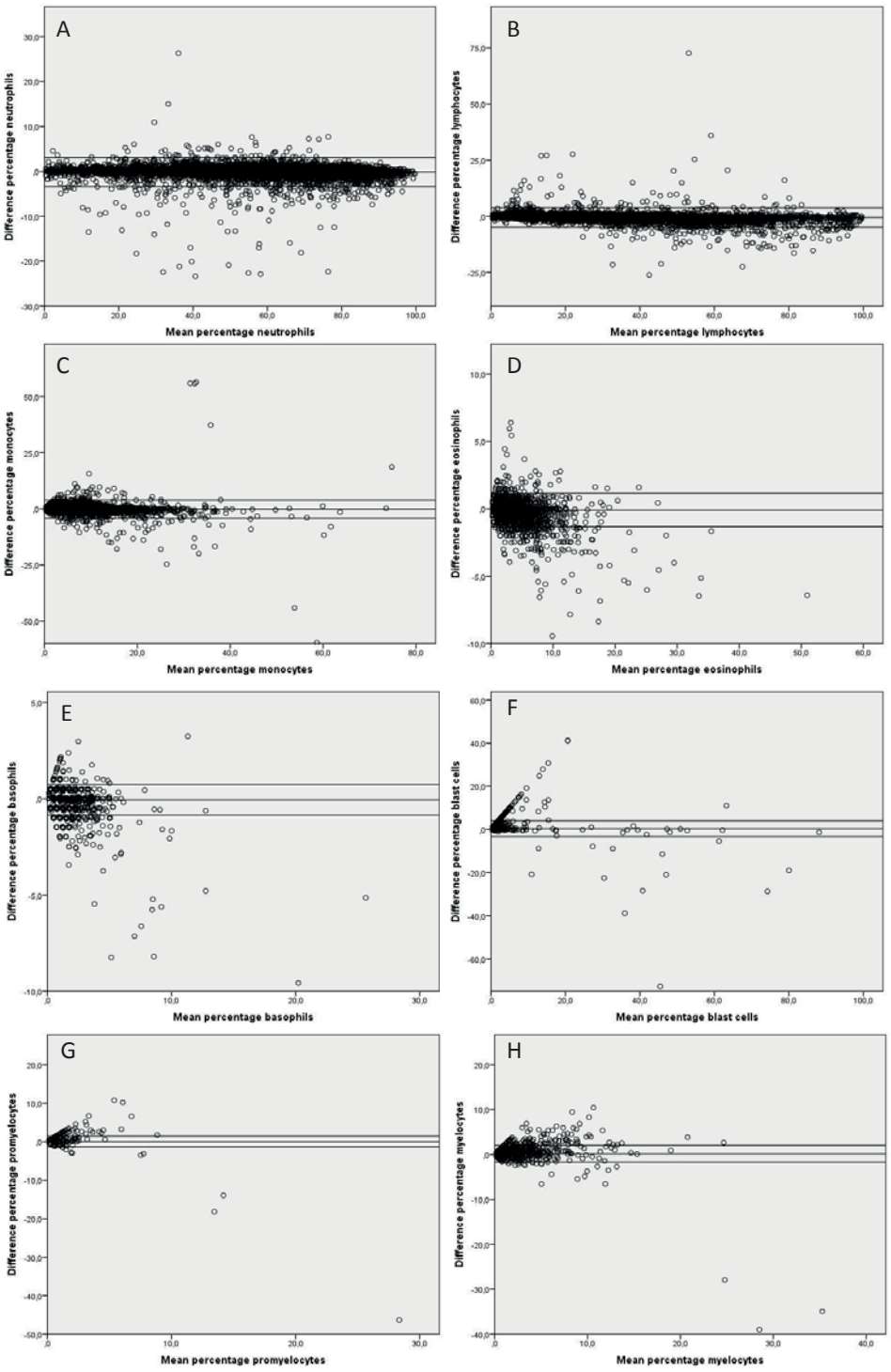
Table 1, *Overview of the correlation, mean difference, limits of agreement, regression equation, and the corresponding 95% confidence intervals (CI) for the intercept and the slope of the regression equation. The classes promonocytes, prolymphocytes, hairy cells, and cleaved cells cannot yet be detected by the DM and were therefore not included in this table. Neutrophils include band and segmented neutrophils. Lymphocytes also included variant lymphocytes. The mean difference and limits of agreement were added to the Bland-Altman plots as reference lines. The regression equation and the corresponding 95% confidence intervals were used to assess constant and proportional bias. Significant biases are highlighted with an asterisk.*

Discussion

Before digital microscopy can be accepted as an improvement over the current manual method and used as a standardized diagnostic tool, it is necessary to establish that the systems are as reliable as manual assessment[4]. A large database of leukocytes was used to compare the pre-classification performance of the DM to morphological experts.

The detection of blast cells is essential for the correct and early diagnosis of patients with haematological malignancies. It is therefore of extreme importance that the DM does not miss any blast cells (i.e. displays a high sensitivity). In the current study, the DM achieved a blast cell sensitivity of 100% and a specificity of 67%. The rather low specificity indicates that, quite often, the DM classifies a leukocyte as a blast cell, while the cell actually belongs to a different class. In several cases, the expert found a higher percentage of blast cells compared with the DM. While the DM shows excellent sensitivity for blast cells, it does remain necessary for the operator to routinely check the exact percentage.

Excellent accuracy was found for the five main cell classes as shown by the small limits of agreement approaching 0, in combination with the high correlation. The mean differences shown in the Bland-Altman plots were small enough to be considered clinically insignificant[10]. However, rare individual cases may show large differences between the


A
Difference percentage neutrophils
Mean percentage neutrophils
B
Difference percentage lymphocytes
Mean percentage lymphocytes
C
Difference percentage monocytes
Mean percentage monocytes
D
Difference percentage eosinophils
Mean percentage eosinophils
E
Difference percentage basophils
Mean percentage basophils
F
Difference percentage blast cells
Mean percentage blast cells
G
Difference percentage promyelocytes
Mean percentage promyelocytes
H
Difference percentage myelocytes
Mean percentage myelocytes

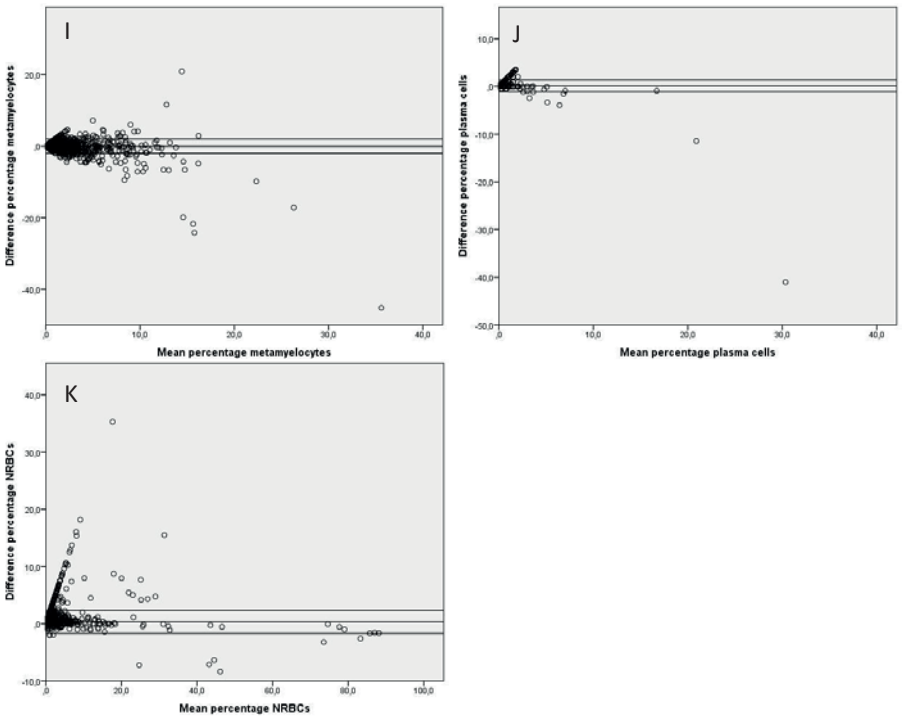


Figure 1, *Bland-Altman plots for A) neutrophils, B) lymphocytes, C) monocytes, D) eosinophils, E) basophils, F) blast cells, G) promyelocytes, H) myelocytes, I) metamyelocytes, J) plasma cells, and K) NRBSc.*

results obtained by the DM and by the expert. In these cases, the DM generally classified cells into one of the five main classes, while the expert classified the cells into one or several of the less common classes.

A statistically significant proportional bias was present for the five main classes, but was too small to be considered clinically relevant, as determined by an experienced haematologist. Constant bias was found for nine classes, including four of the five main classes, but was small enough to be considered negligible. However, when analysing a database of this size, even small biases are statistically significant.

The DM's performance for less common cell classes ranged from adequate (blast cells, correlation = 0.840) to poor (promyelocytes, correlation = 0.432), with small mean differences. However, the majority of samples did not contain these classes according to both the DM and the expert. The calculated mean difference was 0 in these cases, markedly lowering the mean. For these classes, Bland-Altman plots may not be the most fitting method for the analysis of the results. Metamyelocytes showed a clinically significant proportional bias. However, the algorithms used by the DM for this class, and

for the other less common classes, are still in development. Further work is needed to enhance the DM's performance for these classes.

Four rare but clinically significant classes – promonocytes, prolymphocytes, hairy cells, and cleaved cells – cannot yet be detected by the DM. The operator can use the overview option of the DM to review the complete slide after pre-classification to check the DM's performance and detect these rare classes. In practice, the DM can be used as a screening tool for peripheral blood smears, saving time, and reducing workload. The presence of the operator is still required to ensure the proper classification of the less common and rare cell classes.

The next step in morphology will be automated assessment of blood samples, which will allow a blood sample to be processed by a cell counter, an automated preparation unit, and a DM system without any manual intervention, while the results obtained can be sent to the laboratory information system without manual confirmation. This will decrease labour costs (an important issue in today's healthcare system), minimize inter-observer variability, and reduce reporting time for morphological assessment of PBS. The database described here will next be used to assess the possibility of autovalidation of a DM system, which will, if successful, exclude manual interference.

In conclusion, the DM is capable of an excellent performance for the five main blood cell classes and blast cells, but at this moment, manual intervention remains necessary to 'help' the system with the less common classes and the occasional outlier. The algorithms used by the DM to classify the less common classes do require further refinement to improve the DM's pre-classification performance. Nonetheless, the current pre-classification performance of DM systems is a significant step toward the acceptance of DM systems as the standard diagnostic tool for morphological assessment.

References

1. Rumke CL. Imprecision of ratio-derived differential leukocyte counts. *Blood Cells*. 1985;11(2):311-4, 315.
2. Bentley SA. Automated differential white cell counts: A critical appraisal. *Baillieres Clin Haematol*. 1990;3(4):851-869.
3. Ceelie H, Dinkelaar RB, van Gelder W. Examination of peripheral blood films using automated microscopy; evaluation of diffmaster octavia and cellavision DM96. *J Clin Pathol*. 2007;60(1):72-79.
4. Kratz A, Bengtsson HI, Casey JE, et al. Performance evaluation of the CellaVision DM96 system: WBC differentials by automated digital image analysis supported by an artificial neural network. *Am J Clin Pathol*. 2005;124(5):770-781.
5. Briggs C, Longair I, Slavik M, et al. Can automated blood film analysis replace the manual differential? an evaluation of the CellaVision DM96 automated image analysis system. *Int J Lab Hematol*. 2009;31(1):48-60.
6. Lee LH, Mansoor A, Wood B, Nelson H, Higa D, Naugler C. Performance of CellaVision DM96 in leukocyte classification. *J Pathol Inform*. 2013;4:14-3539.114205. Print 2013.
7. Cornet E, Perol JP, Troussard X. Performance evaluation and relevance of the CellaVision DM96 system in routine analysis and in patients with malignant hematological diseases. *Int J Lab Hematol*. 2008;30(6):536-542.
8. Rollins-Raval MA, Raval JS, Contis L. Experience with CellaVision DM96 for peripheral blood differentials in a large multi-center academic hospital system. *J Pathol Inform*. 2012;3:29.
9. Billard M, Lainey E, Armoogum P, Alberti C, Fenneteau O, Da Costa L. Evaluation of the CellaVision DM automated microscope in pediatrics. *Int J Lab Hematol*. 2010;32(5):530-538.
10. Bland JM, Altman DG. Statistical methods for assessing agreement between two methods of clinical measurement. *Lancet*. 1986;1(8476):307-310.

PART III

General discussion and summary

9

General discussion

Anaemia is a significant health problem across the world. It is predominant in the most vulnerable population groups; children below five years of age and the elderly (≥ 65 years)[1,2]. Anaemic children may have issues with mental and motor development[1]. In the elderly anaemia is associated with detrimental effects on many aspects of health, such as an increased risk of recurrent falls[3-5] and more frequent hospital admissions[6-10], and increased mortality rates[7-9,11-25]. Anaemia is considered a symptom of diverse underlying aetiology. The most common causes are anaemia of chronic disease (ACD), iron deficiency anaemia (IDA), renal anaemia, and unknown anaemia (**Chapter 2** and **3**). The less prevalent causes, such as haemolysis and possible bone marrow disease, generally get little attention in population-based anaemia studies. Knowledge regarding their individual impact on survival is lacking as well.

In this thesis, we focused on patients presenting to their general practitioner (GP) with newly diagnosed anaemia. GPs have a central role in the healthcare system, as they usually are the first specialist a patient comes in contact with when seeking medical aid. As such, GPs encounter a wide variety of patients and conditions, and anaemia is a common finding. Clear prevalence rates of a broad range of causes and proper guidelines are necessary to aid physicians with the evaluation of anaemia. We analysed several causes of anaemia, their individual impact on mortality risk and their prevalence rates in an anaemic community-dwelling population (≥ 50 years). We also analysed the predictive value of the mean corpuscular volume (MCV) to determine the validity of the current MCV-based guidelines for the evaluation of anaemia.

Defining anaemia

Anaemia is most commonly defined by a cut-off value of haemoglobin. Most publications use the cut-off values determined by the World Health Organisation (WHO)[1]. However, the WHO standard has become a topic of debate during the past decade since it was determined over 45 years ago in a small number of subjects and does not take potential differences between races into account. Alternative values, based on a larger population database, have been suggested by Beutler *et al*[26], but the WHO standard is still maintained. Our research focused on a population of general practice patients in the Netherlands. Therefore, we used the cut-off values recommended by the Dutch College for General Practitioners (DCGP)[27,28] to define anaemia in this thesis. These values are used in daily practice in the Netherlands, where all described studies were conducted. Its use therefore more accurately reflects day-to-day reality of the patients included in our database, compared to the internationally recognised WHO standard.

Anaemia diagnostics

MCV-based classification of anaemia

Establishing the presence of anaemia is relatively straightforward but determining the underlying cause may be challenging. And yet, elucidating this cause is necessary for the swift and correct treatment of both the anaemia and any potential underlying conditions. Without a clear aetiology, targeted therapy becomes very difficult[29]. Several algorithms have been developed to aid physicians with the evaluation of anaemia[27,30-32]. Most of these algorithms follow the hallmark classification based on MCV. This classification was first postulated by Wintrobe in the 1930s and has remained leading ever since[33]. Despite its extensive use in anaemia evaluation, the actual value of MCV in daily practice has been insufficiently studied. Several relatively small studies in elderly hospitalised patients have shown that MCV does not have any added value for the evaluation of anaemia in this population[34-36].

In **Chapter 2** we analysed the validity of MCV-based algorithms in general practice. A traditional classification emerged from statistical testing but a strict application of these results culminated in almost 30% of causes found in the 'wrong' class. The negative predictive value of MCV was considered too low for six of the causes to be able to reliably exclude them based on MCV alone. This could only be partly explained by the often multifactorial nature of anaemia in this population. These findings were supported by the results in **Chapter 4**, where the analysis of the macrocytic cohort of our population database showed a considerable prevalence of laboratory findings compatible with anaemia of chronic disease (ACD), iron deficiency anaemia (IDA) and renal anaemia. All three causes had not been described in a macrocytic cohort before and should not be found in this cohort according to the traditional classification. Based on this data we concluded that the validity of the use of MCV in the evaluation of anaemia in general practice could not be substantiated.

Future anaemia algorithms

In **Chapters 2** and **4**, we demonstrated that the predictive value of MCV is simply not strong enough to justify excluding causes based solely on this parameter. This leads to the conclusion that the existing MCV-based algorithms for the evaluation of anaemia should be reconsidered. A first step towards developing algorithms without MCV is the new DCGP guideline used in the Netherlands. It puts less emphasis on MCV, but its developers did not venture to remove the parameter entirely.

Aside from removing MCV from the criteria, the new generation of algorithms should incorporate several aspects. There should be a move towards a digital algorithm, in which the results of a standardised laboratory anaemia protocol can be uploaded. The algorithm

can then show a probability score for each cause, giving physicians a good overview of the contributing factors for the patients' anaemia. The protocol should at least contain the following parameters: haemoglobin, ferritin, iron, transferrin, transferrin saturation, C-reactive protein, vitamin B12, folic acid, erythrocytes, thrombocytes, leukocytes, creatinine/MDRD, and LDH. These parameters are most commonly included in the current evaluation of anaemia and laboratories should therefore have no difficulty implementing the digital algorithm. This batch of tests also allows for the diagnosis of a wide range of causes. More recently developed parameters, such as the soluble transferrin receptor, may also be included, if their added worth can be demonstrated. Hepcidin has emerged as a marker capable of distinguishing between ACD and IDA when ferritin is inconclusive[37,38]. However, standardisation of the available tests remains an issue and determining the parameter is currently still quite costly, and not widely available in laboratories. Hepcidin also does not give a decisive test result for patients presenting with both ACD and IDA, which remains a difficult combination to diagnose and treat. This prevents hepcidin from being an appropriate parameter to include in a diagnostic anaemia algorithm at this point in time.

Since anaemia is often multifactorial, especially in the elderly population (**Chapter 2** and **3**)[39], we should move away from the strict 'one cause only' precedent set by the current, non-digitalised algorithms. A digital algorithm will allow for several possible causes per patient and also show corresponding probability scores per cause. To summarise, in the future, a physician should be able to upload the results from a standardised laboratory protocol into an algorithm and receive an overview of the possible causes and the probability of these causes contributing to the present anaemia.

In future research, the population database described in this thesis will be used to create a digitalised, evidence-based algorithm for the evaluation of anaemia. Besides allowing for a multifactorial approach, this algorithm could lower the percentage of unexplained cases of anaemia in general practice. Decreasing the prevalence of unexplained anaemia will facilitate an increase in correctly treated anaemia cases.

Other factors

An earlier review noted that there is no correlation between the severity of anaemia and its underlying causes[40]. This observation is in contrast with our findings in **Chapter 2**. Our data does suggest there is an association between the cause and type (mild, moderate or severe) of anaemia. However, due to the small groups of patients presenting with moderate or severe anaemia, we cannot draw definite conclusions on the correlation between severity and cause from this cohort. Moreover, it is unlikely that the severity of the anaemia will be of use in the determination of the underlying aetiology, since all causes, except unknown anaemia, were found in all three types. The severity does

influence a GP's decision on the course of treatment for the patient. No patient from the severe cohort was found to have an unknown anaemia. GPs responded to such a low haemoglobin level with a swift, often immediate, referral to the hospital for an extensive evaluation (personal observation of author). The association between the severity of anaemia and GP's behaviour could also be seen for the treatment of IDA. GPs were more likely to follow the current recommendations for further diagnostics and treatment in case the anaemia was more severe, as described in **Chapter 6**.

Both renal anaemia and IDA were found more often in women, as shown in **Chapter 2**. For IDA, this could be explained by the large number of women between 50 and 54 years old, who may not have yet gone through menopause. The prevalence of renal anaemia increases with age, due to a decrease in renal function, ascribed to a reduced erythropoietin secretion[41]. It is more common in women, most likely due to the greater proportion of women than men in the two highest age groups 75-84 years and 85+ years (51.3% and 65.7% respectively). ACD, on the other hand, was more often found in men. This has been observed before[2], though this is not a universal result[42] and the exact significance of this finding is unclear. Whether gender will be a relevant factor in comprehensive anaemia diagnostics still needs to be determined. It may influence the actions undertaken by a GP following the initial diagnosis of anaemia and its underlying cause. As seen in **Chapter 6**, GPs are much less inclined to order an endoscopy in female patients with IDA, even though the current guidelines recommend this in cases where hypermenorrhoea has been excluded.

Mortality

Anaemia was long considered to be an innocent consequence of aging and may still be regarded as such in daily practice. To provide a clear understanding of the aetiology of anaemia and the potential consequences, reliable prevalence rates of the possible causes and data on their influence on mortality are necessary. Both the prevalence rates and the association was determined in a large cohort of general practice patients newly diagnosed with anaemia in **Chapter 2** and **3**. Patients with unknown anaemia were used as reference group for the assessment of mortality risk.

No ratio could be determined for haemoglobinopathy (no deaths observed during the follow-up period). Patients diagnosed with one of the other eight causes or with multiple causes did all show an increased mortality risk. These risks reached significance for the categories ACD, possible bone marrow disease, folic acid deficiency, renal anaemia and multiple causes. Associations between anaemia aetiology and mortality have been clearly established, but the underlying mechanisms are less clear. So far, epidemiological studies have been unable to establish a causal link between anaemia itself and functional decline in the elderly[43]. There is also no literature supporting a causal link between anaemia and

survival. It is therefore probable that the significant influence of these categories can be attributed to the underlying conditions. Successfully treating these conditions could therefore potentially reverse the increased risk, though this may be easier said than done. ACD and possible bone marrow disease are regularly caused by malignancies, which can be difficult to treat. Considering the median age of the cohort, further diagnostics or treatment of anaemia in general may be refused or decided against by the patient or GP, due to age, co-morbidities and/or overall health status.

All-cause anaemia was also associated with an increased mortality risk when comparing the anaemic population with the overall Dutch population. This effect was the strongest in the youngest age groups. The presence of severe co-morbidities may have been a possible explanation for this observation. Studies in community-dwelling populations (≥ 65 years) found a higher prevalence of coronary heart disease, congestive heart failure, diabetes, malignancies, infectious disease and kidney disease in anaemic patients[8,9]. However, co-morbidities were not registered in our study, so their potential presence and influence could not be substantiated. In the advanced age groups, it is most likely age itself that has a more significant influence on mortality, lessening the impact of anaemia. In the younger age groups, the impact of anaemia is, therefore, more pronounced.

Chapters 5 and **6** describe the mortality risk associated with adherence and non-adherence to the current guidelines for the treatment of ACD and IDA, respectively. The GPs decision on adherence was not reflected in the mortality risk for ACD patients, but for IDA patients a significant difference was found. Patients to whom the standard was not applied showed a significantly increased mortality risk compared to patients to whom the standard was applied. It should be noted that this survival model was not corrected for co-morbidities. A patient's co-morbidities and overall health status could have significantly influenced the GP's decision to follow the standard and could also have influenced survival.

Further diagnostics

Despite the widespread prevalence of anaemia there is little data available regarding how widely and successfully anaemia is managed by clinicians. The studies that are available, including cohorts of cancer patients and renal transplant recipients, indicate treatment of the decreased haemoglobin level itself is limited[44,45]. Elucidating the underlying cause of anaemia is essential for targeted treatment[29]. However, laboratory measurements do not always provide a clear cause, or the results recommend further invasive diagnostics. For example, the finding of IDA should be followed by an endoscopy unless there is a clear cause for the deficiency, such as hypermenorrhea or blood donation. Guidelines have been set up to document the recommended further diagnostics[27]. We evaluated the adherence to the national guideline for ACD and IDA, respectively (**Chapters 5** and **6**).

Whether any further diagnostics for ACD or IDA are actually initiated by the GP depends on several factors, including the patient's age, the severity of the anaemia, any existing co-morbidities and the patient's wishes regarding their treatment. The IDA patients (described in **Chapter 6**) for whom the standard was not applied may have had a poorer health status or more co-morbidities than the IDA patients for whom the standard was applied. We studied a relatively elderly cohort, and the age of the patient may significantly influence the GP's decision regarding which treatment strategy to follow. The GP may decide against further diagnostics if there are no true benefits to be gained from treatment due to the patients age, health status or co-morbidities. The severity of anaemia may also have an impact. A portion of the patients studied in **Chapter 6** did not receive oral iron supplementation. This supplementation can give severe side effects and GPs may be reluctant to prescribe it if the anaemia is very mild (personal communication). The guideline for ACD requires further investigations to elucidate the underlying condition, if one is not immediately apparent, but these investigations were infrequently ordered. GPs may decide against further investigations if the anaemia is mild and not considered potentially harmful to the patient or if the patient has severe co-morbidities. Again, the patients age or health status may already lead to a decision against treatment, rendering invasive diagnostics irrelevant.

GPs will consider both the risks and the potential benefits of any procedure when deciding on further evaluation of any anaemia. In patients aged 85 years or older, anaemia no longer significantly influences mortality (**Chapter 3**). Especially in this very old population, invasive diagnostics should be used with extreme care, since, with regards to decreasing mortality risks, the gain will be minimal. In all age groups and severity types of anaemia, the patient's wishes will be a major factor in the GP's decisions.

Treatment

It has been suggested that the fatigue, cardiovascular complications and impaired physical performance often seen with anaemia are caused by the reduced oxygen-carrying capacity of the blood[46]. However, at this moment it is unclear whether reversing the anaemia itself (i.e. increasing the haemoglobin level) without also successfully treating the underlying condition can give improvements. Small trials have been conducted to analyse the effects of systematic erythropoietin treatment. Improvements in quality of life and a decreased need for blood transfusions have been reported[47-49], but so have increased risk of thromboembolic events, increased risk of cardiovascular events, and increased mortality[49-53]. Treating mild anaemia with blood transfusions is strongly discouraged[43,54-56] due to increased mortality[57,58], and is, therefore, not an option for the large majority of anaemic patients. Due to the central role of hepcidin in iron metabolism[59], this peptide and its associated pathways have become the target for newly developed therapeutics for iron disorders. Hepcidin agonists, either mimics or stimulators of hepcidin production, may be

beneficial in iron overload disorders such as hemochromatosis and β-thalassemia. Patients with anaemia of chronic disease may benefit from hepcidin inhibitors. These may either inhibit hepcidin production, neutralise the peptide or interfere with the binding between hepcidin and the iron transporter ferroportin[60-62].

Increasing the haemoglobin level without treating the underlying aetiology could be a solution for patients with unexplained anaemia, since in these cases, there is no clear underlying condition to treat. Currently, over 25% of anaemia cases remain unexplained. Unknown anaemia was significantly associated with mild anaemia (**Chapter 2**). Yet even if it is mild, the association of anaemia with detrimental effects on both health and mortality can already be observed[5,21,24,25,63-68]. In addition, unknown anaemia was also associated with an increased mortality risk[42]. Treating the anaemia itself (i.e. the decreased haemoglobin level) without treating the underlying condition, would also potentially help those patients who no longer wish for invasive diagnostics to determine any underlying aetiology or are unable to undergo such diagnostics due to age or severe co-morbidities. Improving their haemoglobin level may have positive effects on, for example, their quality of life.

So far, no satisfactory results have been obtained with the currently available medications (e.g. erythropoietin replacement or blood transfusions) for the correction of the haemoglobin level alone. Therapeutics targeting hepcidin seem promising but need to achieve a very solid safety profile to justify their use in daily practice.

Limitations of population database

All studies described in part I of this thesis use a large, population-based database of general practice patients with newly diagnosed anaemia. Unfortunately, this database does have several limitations. The study has an epidemiological setting and the criteria used to define the different causes of anaemia cannot strictly be regarded as diagnostic, even though the definitions were based on the GP standard in use during the study period and on the knowledge of experienced haematologists, internists and clinical chemists. The observation of a normalised haemoglobin level after treatment of the underlying aetiology is the most reliable way of determining whether or not the initial diagnosis was correct. Since we did not perform a follow-up on the included patients, this confirmation was not available to us. For example, the presence of ACD, IDA and renal anaemia in the macrocytic cohort; a normalisation of both haemoglobin and MCV after treating these conditions would have provided firmer evidence that the laboratory results connected to ACD, IDA and renal anaemia were associated with the observed decrease in haemoglobin and increased MCV.

The diagnosis of anaemia was based on a single haemoglobin measurement. The study protocol did not include a repeat of this measurement. During the follow-up period, a change in the haemoglobin level of a patient may occur. A longer follow-up results in a higher probability of such a change occurring[24]. This highlights one possible drawback for using this database to assess mortality, since anaemia and its underlying cause or causes may have been resolved at the time of death. Therefore, an association between anaemia and mortality can be established, but not a causal link. Nonetheless, the described database is one of the largest-population based databases with anaemic general practice patients and provides a solid basis for further research into anaemia in this population.

Digital microscopy

Laboratory techniques play a vital role in the evaluation of anaemia. One of the key components of such an evaluation is the peripheral blood smear (PBS). Cell counters give an alert when the laboratory measurements are suspect for a bone marrow disease, which is not uncommon in anaemic patients. A PBS is then generated for further evaluation. For decades, morphological assessment of the PBS has been done manually, using a light microscope. This method is prone to inter-observer variability, requires highly and continuously trained personnel and is time-consuming[69-71]. Digital microscope (DM) systems have been developed for the automation and standardisation of morphological assessment. To allow DM systems to become fully accepted as the replacement of manual assessment, the current gold standard, these systems need to be extensively validated. Two steps of the validation process are described in this thesis.

Validation DM systems

Chapter 7 describes the determination of the inter-laboratory reproducibility. Four independent DM systems of the same brand and product type, operated at different locations and by local operators, can achieve comparable results for the main blood cell classes (neutrophils, lymphocytes, monocytes, eosinophils) and blast cells. The variability was high for basophils, an observation also made when comparing manual counts of this cell class[71,72]. This is most likely due to the low prevalence of these cells in the studied samples. The problem is solved by including more samples, which increases the number of counted basophils. **Chapter 8** describes a large database of 1.4 million leukocytes used to compare the DM to the gold standard. The size of the database and the mix of normal and abnormal blood smears allows for the definite confirmation that the DM systems are capable of achieving excellent accuracy for the five main blood cell classes and can attain a sensitivity of 100% for the detection of blast cells. The system is less capable of properly classifying the less common cell classes and will generally classify these into one of the five main classes. Since these less common classes can be clinically significant, the DM still needs to be supported by a manual expert at this point in time.

Future abilities leukocyte morphology

Currently, the DM is used to pre-classify the PBS, after which the manual expert assesses the slide using the overview option of the software and re-classifies the analysed cells where necessary. This workflow saves time and allows for the digital storage of the slide. The future goal is to fully automate the processing of a PBS, including the analysis, without any manual intervention. Currently this is feasible for the five main blood cell classes, but the performance of the DM for the less common classes is lacking. The algorithms used by the DM to recognise the less common cell classes need to be improved, before this completely automated processing can be put into practice. In addition, the DM will have to be tested again with a large database containing normal and abnormal blood smears to confirm, that the less common cell classes are properly recognised and classified. The inter-laboratory variation for these improved systems will need to be determined for all the cell classes, instead of just the five main classes and blast cells. Any future analysis will require larger sample numbers than those included in the study described in **Chapter 7**.

Future perspectives: erythrocyte and bone marrow morphology

A recent development in DM systems is the automated recognition and classification of red blood cell morphology. DM systems can recognise classes such as target cells, teardrop cells, and sickle cells, and abnormalities such as microcytosis[73,74], which allows the DM to aid in the quick recognition of severe conditions. An example is the diagnosis of thrombotic thrombocytopenic purpura (TTP) and haemolytic-uremic syndrome (HUS), which are both characterised by an increase in schistocytes. The cell class is accurately detected by the DM, allowing for the proper recognition of both TTP and HUS[74]. The current software is also capable of recognising inclusions within erythrocytes such as malaria parasites. The red blood cell module on the DM is currently undergoing validation for use in daily practice as a support for morphology experts. The first results are promising, but are indicating that the software requires further development, for example to improve the differentiation between observed inclusions.

Another aspect of morphology assessment is the analysis of a bone marrow sample through the light microscope. The DM module that should allow for the automated analysis of these samples is currently in development. The main issue appears to be ‘teaching’ the system where to look in the smear produced from the bone marrow sample. Scanning the slide requires a lot of computational power and takes a lot of time. These problems will have to be solved before the bone marrow module can become a valuable addition to the diagnostic workflow.

Conclusions

We analysed a large cohort of general practice patients (≥ 50 years) with newly diagnosed anaemia. Our results show MCV is an unreliable parameter when excluding causes of anaemia or deciding on further diagnostics for the evaluation of anaemia. MCV should, therefore, lose its central role in anaemia evaluation. In case the GP suspects the presence of anaemia, a standardised protocol should be ordered instead of allowing MCV to be leading in any diagnostic decisions. The association between anaemia and mortality was again confirmed. For the first time the impact of less prevalent causes was established. Whether this association is seen because anaemia is a marker of underlying disease or because there exists a more causal connection between anaemia and mortality, remains to be determined.

Digital microscopy has proven itself to be a reliable tool for the analysis of the peripheral blood smear. Considering the continuing development of these systems and the advancing digitalisation of the laboratory workplace, digital microscopy will become a viable alternative for manual assessment and, in time, replace the human component.

The association of anaemia with detrimental effects on health and mortality has been extensively documented. It is now time to study possible treatment options and to determine whether actively treating anaemia itself is a viable option for improving patients' prospects. A thorough evaluation of anaemia will remain necessary, as quickly determining the underlying aetiology gives access to rapid and proper treatment. Future options for this evaluation include developing a digitalised algorithm and the further automation and standardisation of the morphological analysis of leukocytes, erythrocytes and thrombocytes. These options and increased awareness of the detrimental effects of anaemia will hopefully ensure that anaemia will no longer be considered an innocent bystander, but will receive the full attention it requires.

References

1. Kassebaum NJ, Jasrasaria R, Naghavi M, et al. A systematic analysis of global anemia burden from 1990 to 2010. *Blood*. 2014;123(5):615-624.
2. Guralnik JM, Eisenstaedt RS, Ferrucci L, Klein HG, Woodman RC. Prevalence of anemia in persons 65 years and older in the united states: Evidence for a high rate of unexplained anemia. *Blood*. 2004;104(8):2263-2268.
3. Dharmarajan TS, Norkus EP. Mild anemia and the risk of falls in older adults from nursing homes and the community. *J Am Med Dir Assoc*. 2004;5(6):395-400.
4. Guse CE, Porinsky R. Risk factors associated with hospitalization for unintentional falls: Wisconsin hospital discharge data for patients aged 65 and over. *WMJ*. 2003;102(4):37-42.
5. Penninx BW, Pluijm SM, Lips P, et al. Late-life anemia is associated with increased risk of recurrent falls. *J Am Geriatr Soc*. 2005;53(12):2106-2111.
6. Artz AS, Fergusson D, Drinka PJ, et al. Prevalence of anemia in skilled-nursing home residents. *Arch Gerontol Geriatr*. 2004;39(3):201-206.
7. Anand I, McMurray JJ, Whitmore J, et al. Anemia and its relationship to clinical outcome in heart failure. *Circulation*. 2004;110(2):149-154.
8. Penninx BW, Pahor M, Woodman RC, Guralnik JM. Anemia in old age is associated with increased mortality and hospitalization. *J Gerontol A Biol Sci Med Sci*. 2006;61(5):474-479.
9. Culleton BF, Manns BJ, Zhang J, Tonelli M, Klarenbach S, Hemmelgarn BR. Impact of anemia on hospitalization and mortality in older adults. *Blood*. 2006;107(10):3841-3846.
10. Dharmarajan TS, Pankratov A, Morris E, et al. Anemia: Its impact on hospitalizations and length of hospital stay in nursing home and community older adults. *J Am Med Dir Assoc*. 2008;9(5):354-359.
11. da Silveira AD, Ribeiro RA, Rossini AP, et al. Association of anemia with clinical outcomes in stable coronary artery disease. *Coron Artery Dis*. 2008;19(1):21-26.
12. Dunlay SM, Weston SA, Redfield MM, Killian JM, Roger VL. Anemia and heart failure: A community study. *Am J Med*. 2008;121(8):726-732.
13. Groenveld HF, Januzzi JL, Damman K, et al. Anemia and mortality in heart failure patients a systematic review and meta-analysis. *J Am Coll Cardiol*. 2008;52(10):818-827.
14. Go AS, Yang J, Ackerson LM, et al. Hemoglobin level, chronic kidney disease, and the risks of death and hospitalization in adults with chronic heart failure: The anemia in chronic heart failure: Outcomes and resource utilization (ANCHOR) study. *Circulation*. 2006;113(23):2713-2723.
15. Maggioni AP, Opasich C, Anand I, et al. Anemia in patients with heart failure: Prevalence and prognostic role in a controlled trial and in clinical practice. *J Card Fail*. 2005;11(2):91-98.
16. Caro JJ, Salas M, Ward A, Goss G. Anemia as an independent prognostic factor for survival in patients with cancer: A systemic, quantitative review. *Cancer*. 2001;91(12):2214-2221.
17. Ofsthun N, Labrecque J, Lacson E, Keen M, Lazarus JM. The effects of higher hemoglobin levels on mortality and hospitalization in hemodialysis patients. *Kidney Int*. 2003;63(5):1908-1914.
18. Robinson BM, Joffe MM, Berns JS, Pisoni RL, Port FK, Feldman HI. Anemia and mortality in hemodialysis patients: Accounting for morbidity and treatment variables updated over time. *Kidney Int*. 2005;68(5):2323-2330.
19. Landi F, Russo A, Danese P, et al. Anemia status, hemoglobin concentration, and mortality in nursing home older residents. *J Am Med Dir Assoc*. 2007;8(5):322-327.
20. Kikuchi M, Inagaki T, Shinagawa N. Five-year survival of older people with anemia: Variation with hemoglobin concentration. *J Am Geriatr Soc*. 2001;49(9):1226-1228.
21. Chaves PH, Semba RD, Leng SX, et al. Impact of anemia and cardiovascular disease on frailty status of community-dwelling older women: The women's health and aging studies I and II. *J Gerontol A Biol Sci Med Sci*. 2005;60(6):729-735.
22. Izaks GJ, Westendorp RG, Knook DL. The definition of anemia in older persons. *JAMA*. 1999;281(18):1714-1717.
23. Dong X, Mendes de Leon C, Artz A, Tang Y, Shah R, Evans D. A population-based study of hemoglobin, race, and mortality in elderly persons. *J Gerontol A Biol Sci Med Sci*. 2008;63(8):873-878.

24. Riva E, Tettamanti M, Mosconi P, et al. Association of mild anemia with hospitalization and mortality in the elderly: The health and anemia population-based study. *Haematologica*. 2009;94(1):22-28.
25. Teng TH, Finn J, Hung J. Mild anaemia is associated with increased all-cause mortality in heart failure. *Heart Lung Circ*. 2010;19(1):31-37.
26. Beutler E, Waalen J. The definition of anemia: What is the lower limit of normal of the blood hemoglobin concentration? *Blood*. 2006;107(5):1747-1750.
27. Van Wijk MAM, Mel M, Muller PA, Zilverentand WGJ, Pijnenborg L, Kolnaar BGM. NHG-standaard anemie. *Huisarts & Wetenschap*. 2003;46:21-29.
28. NHG-werkgroep anemie. NHG standaard anemie (eerste herziening). *Huisarts & Wetenschap*. 2014;57(10):528-536.
29. Price EA, Mehra R, Holmes TH, Schrier SL. Anemia in older persons: Etiology and evaluation. *Blood Cells Mol Dis*. 2011;46(2):159-165.
30. Bross MH, Soch K, Smith-Knuppel T. Anemia in older persons. *Am Fam Physician*. 2010;82(5):480-487.
31. Kaferle J, Strzoda CE. Evaluation of macrocytosis. *Am Fam Physician*. 2009;79(3):203-208.
32. Oosterhuis WP, van der Horst M, van Dongen K, Ulenkate HJ, Volmer M, Wulkan RW. Prospective comparison of the flow chart for laboratory investigations for anaemia from the dutch college of general practitioners' guideline 'anaemia' with a self-developed, substantive and logistical alternative flow chart. *Ned Tijdschr Geneeskd*. 2007;151(42):2326-2332.
33. Wintrobe MM. Anemia classification and treatment on the basis ofdifferences in the average volume and hemoglobin content of the red corpuscles. *Archives of Internal Medicine*. 1934;54(2):256-280.
34. Seward SJ, Safran C, Marton KI, Robinson SH. Does the mean corpuscular volume help physicians evaluate hospitalized patients with anemia? *J Gen Intern Med*. 1990;5(3):187-191.
35. Petrosyan I, Blaison G, Andres E, Federici L. Anaemia in the elderly: An aetiologic profile of a prospective cohort of 95 hospitalised patients. *Eur J Intern Med*. 2012;23(6):524-528.
36. Bach V, Schruckmayer G, Sam I, Kemmler G, Stauder R. Prevalence and possible causes of anemia in the elderly: A cross-sectional analysis of a large european university hospital cohort. *Clin Interv Aging*. 2014;9:1187-1196.
37. D'Angelo G. Role of hepcidin in the pathophysiology and diagnosis of anemia. *Blood Res*. 2013;48(1):10-15.
38. Vermeulen E, Vermeersch P. Hepcidin as a biomarker for the diagnosis of iron metabolism disorders: A review. *Acta Clin Belg*. 2012;67(3):190-197.
39. Merlo C, Wuillemin WA. Prevalence and causes of anemia in a city general practice. *Praxis (Bern 1994)*. 2008;97(13):713-718.
40. Andres E, Serraj K, Federici L, Vogel T, Kaltenbach G. Anemia in elderly patients: New insight into an old disorder. *Geriatr Gerontol Int*. 2013;13(3):519-527.
41. Ble A, Fink JC, Woodman RC, et al. Renal function, erythropoietin, and anemia of older persons: The InCHIANTI study. *Arch Intern Med*. 2005;165(19):2222-2227.
42. Shavelle RM, MacKenzie R, Paculdo DR. Anemia and mortality in older persons: Does the type of anemia affect survival? *Int J Hematol*. 2012;95(3):248-256.
43. Merchant AA, Roy CN. Not so benign haematology: Anaemia of the elderly. *Br J Haematol*. 2012;156(2):173-185.
44. Verbeke N, Beguin Y, Wildiers H, et al. High prevalence of anaemia and limited use of therapy in cancer patients: A belgian survey (anaemia day 2008). *Support Care Cancer*. 2012;20(1):23-28.
45. Molnar MZ, Mucsi I, Macdougall IC, et al. Prevalence and management of anaemia in renal transplant recipients: Data from ten european centres. *Nephron Clin Pract*. 2011;117(2):c127-34.
46. Semba RD, Ricks MO, Ferrucci L, et al. Types of anemia and mortality among older disabled women living in the community: The women's health and aging study I. *Aging Clin Exp Res*. 2007;19(4):259-264.
47. Agnihotri P, Telfer M, Butt Z, et al. Chronic anemia and fatigue in elderly patients: Results of a randomized, double-blind, placebo-controlled, crossover exploratory study with epoetin alfa. *J Am Geriatr Soc*. 2007;55(10):1557-1565.

48. Ershler WB, Artz AS, Kandahari MM. Recombinant erythropoietin treatment of anemia in older adults. *J Am Geriatr Soc*. 2001;49(10):1396-1397.
49. Tonelli M, Hemmelgarn B, Reiman T, et al. Benefits and harms of erythropoiesis-stimulating agents for anemia related to cancer: A meta-analysis. *CMAJ*. 2009;180(11):E62-71.
50. Bohlius J, Schmidlin K, Brillant C, et al. Recombinant human erythropoiesis-stimulating agents and mortality in patients with cancer: A meta-analysis of randomised trials. *Lancet*. 2009;373(9674):1532-1542.
51. Phrommintikul A, Haas SJ, Elsik M, Krum H. Mortality and target haemoglobin concentrations in anaemic patients with chronic kidney disease treated with erythropoietin: A meta-analysis. *Lancet*. 2007;369(9559):381-388.
52. Rosenzweig MQ, Bender CM, Lucke JP, Yasko JM, Brufsky AM. The decision to prematurely terminate a trial of R-HuEPO due to thrombotic events. *J Pain Symptom Manage*. 2004;27(2):185-190.
53. Wun T, Law L, Harvey D, Sieracki B, Scudder SA, Ryu JK. Increased incidence of symptomatic venous thrombosis in patients with cervical carcinoma treated with concurrent chemotherapy, radiation, and erythropoietin. *Cancer*. 2003;98(7):1514-1520.
54. Goddard AF, James MW, McIntyre AS, Scott BB, British Society of Gastroenterology. Guidelines for the management of iron deficiency anaemia. *Gut*. 2011;60(10):1309-1316.
55. Pasricha SR, Flecknoe-Brown SC, Allen KJ, et al. Diagnosis and management of iron deficiency anaemia: A clinical update. *Med J Aust*. 2010;193(9):525-532.
56. Van Puyvelde K, Cytryn E, Mets T, Beyer I. Anaemia in the elderly. *Acta Clin Belg*. 2009;64(4):292-302.
57. Chatterjee S, Wetterslev J, Sharma A, Lichstein E, Mukherjee D. Association of blood transfusion with increased mortality in myocardial infarction: A meta-analysis and diversity-adjusted study sequential analysis. *JAMA Intern Med*. 2013;173(2):132-139.
58. Paone G, Likosky DS, Brewer R, et al. Transfusion of 1 and 2 units of red blood cells is associated with increased morbidity and mortality. *Ann Thorac Surg*. 2014;97(1):87-93; discussion 93-4.
59. Ganz T. Hepcidin, a key regulator of iron metabolism and mediator of anemia of inflammation. *Blood*. 2003;102(3):783-788.
60. Ganz T, Nemeth E. The hepcidin-ferroportin system as a therapeutic target in anemias and iron overload disorders. *Hematology Am Soc Hematol Educ Program*. 2011;2011:538-542.
61. Fung E, Nemeth E. Manipulation of the hepcidin pathway for therapeutic purposes. *Haematologica*. 2013;98(11):1667-1676.
62. Ruchala P, Nemeth E. The pathophysiology and pharmacology of hepcidin. *Trends Pharmacol Sci*. 2014;35(3):155-161.
63. Penninx BW, Pahor M, Cesari M, et al. Anemia is associated with disability and decreased physical performance and muscle strength in the elderly. *J Am Geriatr Soc*. 2004;52(5):719-724.
64. Penninx BW, Guralnik JM, Onder G, Ferrucci L, Wallace RB, Pahor M. Anemia and decline in physical performance among older persons. *Am J Med*. 2003;115(2):104-110.
65. Chaves PH, Carlson MC, Ferrucci L, Guralnik JM, Semba R, Fried LP. Association between mild anemia and executive function impairment in community-dwelling older women: The women's health and aging study II. *J Am Geriatr Soc*. 2006;54(9):1429-1435.
66. Thein M, Ershler WB, Artz AS, et al. Diminished quality of life and physical function in community-dwelling elderly with anemia. *Medicine (Baltimore)*. 2009;88(2):107-114.
67. Lucca U, Tettamanti M, Mosconi P, et al. Association of mild anemia with cognitive, functional, mood and quality of life outcomes in the elderly: The "health and anemia" study. *PLoS One*. 2008;3(4):e1920.
68. Hong CH, Falvey C, Harris TB, et al. Anemia and risk of dementia in older adults: Findings from the health ABC study. *Neurology*. 2013;81(6):528-533.
69. Rumke CL. Imprecision of ratio-derived differential leukocyte counts. *Blood Cells*. 1985;11(2):311-4, 315.
70. Bentley SA. Automated differential white cell counts: A critical appraisal. *Baillieres Clin Haematol*. 1990;3(4):851-869.
71. Ceelie H, Dinkelaar RB, van Gelder W. Examination of peripheral blood films using automated microscopy; evaluation of diffmaster octavia and cellavision DM96. *J Clin Pathol*. 2007;60(1):72-79.

72. Briggs C, Longair I, Slavik M, et al. Can automated blood film analysis replace the manual differential? an evaluation of the CellaVision DM96 automated image analysis system. *Int J Lab Hematol*. 2009;31(1):48-60.
73. Egelé A, van Gelder W, Riedl J. Automated detection and classification of teardrop cells by a novel RBC module using digital imaging/microscopy.. *International Journal of Laboratory Haematology*. 2015.
74. Egelé A, van Gelder W, Riedl J. Automated detection and classifcation of schistocytes by a novel red blood cell module using digital imaging/microscopy. *Journal of Hematology*. 2015;4(2):184-186.

10

Summary

Anaemia is a leading health problem worldwide with a high burden in the most vulnerable populations: children below five years of age and people aged 85 years and above [1,2]. It is generally defined by a decreased haemoglobin level and was long considered an innocent consequence of aging in the elderly population. However, data on the association of anaemia with detrimental effects on health aspects, quality of life and survival have been steadily accumulating over the past decade. Currently, there are only a few options for the treatment of the decreased haemoglobin level itself. To alleviate the anaemia, targeted treatment of the underlying aetiology is necessary. Reliable prevalence rates for the different causes of anaemia and decisive diagnostic guidelines are important aids for the physician with the diagnosis and treatment of anaemia and its aetiology.

In **Part I** of this thesis we described the extensive analysis of a large cohort of general practice patients newly diagnosed with anaemia. **Chapter 2** provides reliable prevalence rates for nine different causes and for unknown or unexplained anaemia. The distribution of these ten categories of aetiology over the different types of anaemia (mild, moderate or severe, based on haemoglobin), age groups (50-64, 65-74, 75-84 and 85+ years), genders, and classes of anaemia (microcytic, normocytic or macrocytic, based on mean corpuscular volume (MCV)) was evaluated. The most striking results included the high prevalence of iron deficiency in patients with a severe anaemia and the statistically significant increase in the prevalence of renal anaemia with every age group. Current algorithms for the evaluation of anaemia follow a classification based on MCV but the validity of this classification has been challenged. The data in **Chapter 2** confirmed that, in general practice, the predictive value of MCV is not strong enough to justify excluding causes based solely on this parameter. Therefore, existing MCV-based algorithms for the evaluation of anaemia should be reconsidered.

The impact of the aetiology on the mortality risk of anaemic general practice patients was analysed in **Chapter 3**. All causes except haemoglobinopathy (no recorded deaths) were associated with an increased mortality risk. This increase reached statistical significance for the categories anaemia of chronic disease (ACD), possible bone marrow disease, folic acid deficiency, renal anaemia and multiple causes and is most likely due to underlying conditions associated with these causes, such as malignancies. The standardised mortality ratios (SMR) were calculated per five-year age group and gender by comparing the mortality risks of the complete anaemic cohort (all-cause anaemia) and the general Dutch population. Especially in the younger age groups, anaemia was associated with a severe impact on mortality risk in both men and women. From age 85 years and older, anaemia was no longer associated with an increased risk. In this population, invasive diagnostics to determine the underlying cause of anaemia should be used with great restraint. The risks may outweigh the benefits and it is unclear if any improvement in overall survival can be achieved.

The macrocytic cohort of the population database was analysed separately and described in **Chapter 4**. ACD, iron deficiency anaemia (IDA) and renal anaemia were frequently found in this cohort, though these causes are not traditionally described as macrocytic. This finding further supports the results found in **Chapter 2**, concerning the weak predictive value of MCV in general practice. The influence of six different categories of causes on the mortality risk of this cohort was assessed. Univariate analyses showed the lowest 5-year survival rate in patients with renal anaemia, followed by nutrient deficiency (iron, vitamin B12 and folic acid), ACD, multiple causes, and other causes (haemolysis, documented alcohol abuse, possible bone marrow disease, and other). Patients with unknown anaemia had the highest 5-year survival rate. Multivariate analysis was also performed but no significant results were found.

There is little information on the treatment strategies for anaemia applied in daily practice[3,4]. Aetiologies such as ACD and IDA require further diagnostics since the possible underlying conditions for these categories are diverse and extensive. The adherence of general practitioners in the Netherlands to the national guidelines for ACD and IDA was analysed in **Chapters 5** and **6**. As seen in **Chapter 5**, the guideline was applied in the majority of ACD cases, but oral iron supplementation, which is discouraged, was still prescribed in a substantial number of patients. While underlying conditions could not always be established, even after further diagnostics, a survival analysis showed no difference between the survival of patients with or without an established underlying condition. As described in **Chapter 6**, adherence to the guidelines for IDA was seen in a minority of patients. An increased mortality risk was found for patients in whom the guideline was not applied. General practitioners may have decided against following the guideline, which includes a potential risky endoscopy, based on factors such as age, co-morbidities, health status and the patient's wishes. Apart from age, these factors were not recorded, and could not be included in our statistical survival model.

The morphological assessment of the peripheral blood smear remains an important part of laboratory diagnostics. For decades this assessment has been done manually using a light microscope. During the last decade, digital microscopy (DM) systems have developed into reliable diagnostic tools. DM systems allow for an automated and standardised morphological assessment but do need to be extensively validated before they can be accepted as the standard diagnostic tool in blood morphology.

Two steps in the validation process of DM systems are described in **Chapters 7** and **8**. In **Chapter 7** the inter-laboratory variation was determined. Independently operated DM systems yield reproducible pre-classification results when classifying neutrophils, lymphocytes, monocytes, and eosinophils. Blast cells were also detected correctly and with only minor variation between the systems. The pre-classification performance for basophils showed considerable inter-laboratory variation, which was most likely due to

the low incidence of these cells in the used samples. We described the analysis of a large database consisting of 1.4 million leukocytes in **Chapter 8**. Using this database, we determined the accuracy of the DM system for the classification of a wide range of leukocyte classes. The DM system showed an excellent accuracy for the five main cell classes: neutrophils, lymphocytes, monocytes, eosinophils, and basophils. Blast cells were detected with 100% sensitivity. The detection of the less common cell classes (promyelocytes, myelocytes, metamyelocytes, and plasma cells) was less accurate. The rare cell classes (promonocytes, prolymphocytes, hairy cells, and cleaved cells) cannot yet be detected by the DM system. Therefore, manual intervention remains necessary at this point in time to 'help' the system with the less common and rare classes as well as the occasional outlier. The current pre-classification performance of DM systems is a significant step towards the acceptance of these systems as the standard diagnostic tool for morphological assessment.

Anemie is een van de meest voorkomende gezondheidsproblemen in de wereld met een hoge prevalentie in de meest kwetsbare populaties, namelijk kinderen jonger dan vijf jaar en mensen van 85 jaar en ouder[1,2]. Het wordt gekarakteriseerd door een verlaagde concentratie hemoglobine. In de oudere populatie werd anemie lang gezien als een onschuldige consequentie van het ouder worden, maar de bewijzen voor de associatie tussen anemie en schadelijke effecten op gezondheid, kwaliteit van leven en mortaliteit stapelen zich op. Op dit moment zijn er beperkte opties voor het behandelen van de verlaagde concentratie hemoglobine zelf. Om anemie te verhelpen is een gerichte behandeling van de onderliggende etiologie noodzakelijk. Betrouwbare prevalentie cijfers voor de verschillende oorzaken en betrouwbare richtlijnen zijn belangrijke hulpmiddelen voor de arts bij de diagnose en behandeling van anemie en de onderliggende oorzaken.

Deel I van dit proefschrift beschrijft de uitgebreide analyse van een groot cohort van huisartspatiënten met een nieuw gediagnosticeerde anemie. **Hoofdstuk 2** geeft betrouwbare prevalentie cijfers voor negen verschillende oorzaken en voor onbekende anemie. De verdeling van deze tien etiologie categorieën over de verschillende typen anemie (mild, matig en ernstig, gebaseerd op de hemoglobine concentratie), leeftijdsgroepen, (50-64, 65-74, 75-84 en 85+ jaar), geslacht en klassen van anemie (microcytair, normocytair en macrocytair, gebaseerd op het mean corpuscular volume (MCV)) werd geanalyseerd. Twee van de meest opvallende resultaten waren de hoge prevalentie van ijzergebreksanemie in patiënten met een ernstige anemie en de significante verhoging van de prevalentie van renale anemie bij elke leeftijdsgroep. De huidige algoritmes voor de evaluatie van anemie volgen een classificatie gebaseerd op MCV maar de validiteit van deze classificatie wordt tegenwoordig in twijfel getrokken. De resultaten in **Hoofdstuk 2** bevestigen dat de voorspellende waarde van MCV in de huisartsenpraktijk niet sterk genoeg is om oorzaken uit te sluiten op enkel deze parameter. Daarom moeten de bestaande, op MCV gebaseerde algoritmes voor de evaluatie van anemie herzien worden.

De invloed van de etiologie op het mortaliteitsrisico van anemische huisartspatiënten wordt geanalyseerd in **Hoofdstuk 3**. Alle oorzaken, behalve hemoglobinopathie (geen geregistreerde sterfgevallen), werden geassocieerd met een verhoogd mortaliteitsrisico. Deze verhoging was significant voor de oorzaken anemie der chronische ziekten (ACD), mogelijke beenmergziekte, foliumzuur deficiëntie, renale anemie en de categorie meerdere oorzaken. Dit is hoogstwaarschijnlijk het gevolg van onderliggende condities die met deze oorzaken geassocieerd zijn, zoals maligniteiten. De standardised mortality ratio's (SMR) werden berekend per leeftijdsgroep van vijf jaar en geslacht door het mortaliteitsrisico van het complete anemische cohort (alle oorzaken) te vergelijken met het risico van de algemene Nederlandse populatie. Vooral in de jongere leeftijdsgroepen werd anemie geassocieerd met een ernstige impact op het mortaliteitsrisico van zowel mannen en vrouwen. Bij patiënten van 85 jaar en ouder werd dit verhoogde risico niet

langer gezien. In deze populatie moet invasieve diagnostiek ten einde het vaststellen van de onderliggende oorzaak, dan ook met grote terughoudendheid toegepast worden.

In **Hoofdstuk 4** wordt het macrocytaire cohort van de populatie database apart geanalyseerd. Anemie der chronische ziekte, ijzerdeficiëntie anemie en renale anemie werden frequent gevonden in dit cohort, terwijl deze oorzaken traditioneel niet als macrocytair worden beschouwd. Deze resultaten ondersteunen de bevindingen in **Hoofdstuk 2**, aangaande de zwakke voorspellende waarde van MCV in de huisartsenpraktijk. De invloed van zes verschillende categorieën op het mortaliteitsrisico werd onderzocht in dit cohort. Univariaat analyses lieten de laagste 5-jaars overleving zien bij patiënten met renale anemie, gevolgd door nutriënt deficiëntie (ijzer, vitamine B12 en foliumzuur), anemie der chronische ziekten, meerdere oorzaken, en overige oorzaken (hemolyse, gedocumenteerd alcoholmisbruik, mogelijke beenmergziekte en overig). Patiënten met onbekende anemie hadden de hoogste 5-jaars overleving. Multivariaat analyse werd ook uitgevoerd maar gaf geen significante resultaten.

Er is weinig informatie beschikbaar over de behandeling van anemie in de dagelijkse praktijk[3,4]. Oorzaken zoals ACD en ijzerdeficiëntie anemie (IDA) vereisen verdere diagnostiek aangezien de mogelijke onderliggende etiologie voor deze categorieën erg divers is. In **Hoofdstuk 5** en **6** wordt geanalyseerd hoe goed huisartsen de nationale richtlijnen aangaande deze twee oorzaken volgen. Zoals te lezen in **Hoofdstuk 5** werd de richtlijn in de meerderheid van ACD patiënten toegepast, al werd er, tegen de richtlijn in, nog steeds ijzersuppletie voorgeschreven bij een aanzienlijk deel van de patiënten. Een onderliggende oorzaak werd niet altijd gevonden, ondanks verdere diagnostiek. Een overlevingsmodel liet geen verschil zien tussen de overleving van patiënten met en zonder een vastgestelde onderliggende oorzaak. In **Hoofdstuk 6** wordt beschreven dat de richtlijnen voor de behandeling van IDA in een minderheid van patiënten toegepast werd. Een verhoogd mortaliteitsrisico werd gevonden voor de patiënten bij wie de richtlijn niet gevolgd werd. Huisartsen kunnen ervoor gekozen hebben de richtlijn, inclusief een mogelijk risicovolle endoscopie, niet te volgen vanwege factoren zoals leeftijd, co-morbiditeit, gezondheid en wensen van de patiënt. Op leeftijd na, konden deze factoren niet meegenomen worden in ons overlevingsmodel.

De morfologische beoordeling van een perifere bloeduitstrijk blijft een belangrijk onderdeel van laboratorium diagnostiek. Decennialang werd deze beoordeling manueel uitgevoerd met behulp van de lichtmicroscoop. Tijdens het laatste decennium zijn digitale microscopie (DM) systemen ontwikkeld tot betrouwbare diagnostische hulpmiddelen. DM systemen zorgen voor een geautomatiseerde en gestandaardiseerde morfologische beoordeling maar moeten een uitgebreide validatie ondergaan voordat ze geaccepteerd kunnen worden als de standaard diagnostiek op dit gebied.

Twee stappen van dit validatie proces worden beschreven in **Hoofdstuk 7** en **8**. In **Hoofdstuk 7** is de inter-laboratorium variabiliteit vastgesteld. DM systemen gaven onafhankelijk van elkaar reproduceerbare pre-classificatie resultaten bij de classificatie van neutrofielen, lymfocyten, monocyten, en eosinofielen. Blast cellen worden ook correct gedetecteerd en met een minimale variatie. De pre-classificatie van basofielen liet wel een flinke inter-laboratorium variatie zien. Dit was hoogstwaarschijnlijk het gevolg van de lage incidentie van deze cel klasse in de gebruikte monsters. In **Hoofdstuk 8** beschrijven we de analyse van een grote database met 1.4 miljoen leukocyten. Met behulp van deze database werd de nauwkeurigheid van een DM systeem bepaald voor een heel scala aan leukocyten klassen. De DM liet een zeer goede nauwkeurigheid zien voor de vijf hoofdklassen neutrofielen, lymfocyten, monocyten, eosinofielen, en basofielen. Blast cellen werden gedetecteerd met een sensitiviteit van 100%. De detectie van de minder vaak voorkomende klassen promyelocyten, myelocyten, metamyelocyten en plasmacellen was minder nauwkeurig. De zeldzame klassen promonocyten, prolymphocyten, hairy cellen en cleaved cellen kunnen nog niet gedetecteerd worden door het DM systeem. Manuele interventie blijft daarom noodzakelijk op dit moment om het systeem te ‘helpen’ met de minder vaak voorkomende en zeldzame klassen en de incidentele uitschieter. De huidige pre-classificatie van DM systemen is een significante stap richting de acceptatie van deze systemen als het standaard diagnostische hulpmiddel bij morfologische beoordeling.

References

1. Kassebaum NJ, Jasrasaria R, Naghavi M, et al. A systematic analysis of global anemia burden from 1990 to 2010. *Blood*. 2014;123(5):615-624.
2. Guralnik JM, Eisenstaedt RS, Ferrucci L, Klein HG, Woodman RC. Prevalence of anemia in persons 65 years and older in the united states: Evidence for a high rate of unexplained anemia. *Blood*. 2004;104(8):2263-2268.
3. Molnar MZ, Mucsi I, Macdougall IC, et al. Prevalence and management of anaemia in renal transplant recipients: Data from ten european centres. *Nephron Clin Pract*. 2011;117(2):c127-34.
4. Verbeke N, Beguin Y, Wildiers H, et al. High prevalence of anaemia and limited use of therapy in cancer patients: A belgian survey (anaemia day 2008). *Support Care Cancer*. 2012;20(1):23-28.

Portfolio

Training:

- EHA tutorial: Anaemia of Chronic Disease and Myelodysplastic Syndromes (2015)
- Course: Scientific Integrity (2015)
- Course: Good Clinical Practice (2014)

Oral presentations:

- Riedl JA, **Stouten K,** Ceelie H, Boonstra J, Levin MD, van Gelder W. Blood morphology using the digital microscope. Presented at: 10th Dutch Hematology Congress; 2016 January 20-22; Arnhem, Netherlands
- **Stouten K,** Riedl JA, van Houten R, van Rosmalen J, Sonneveld P, Levin MD. Anaemia in general practice: causes and mortality. Presented at: 10th Dutch Hematology Congress; 2016 January 20-22; Arnhem, Netherlands
- **Stouten K,** Schop A, Riedl JA, van Houten R, Levin MD. A new guideline for the evaluation of anaemia: impact on hospital referrals. Presented at: 10th Dutch Hematology Congress; 2016 January 20-22; Arnhem, Netherlands
- **Stouten K,** Riedl JA, Sonneveld P, Levin MD. Anaemia in general practice: causes and survival. Presented at: 9th Dutch Hematology Congress; 2015 January 21-23; Arnhem, Netherlands
- **Stouten K,** Riedl JA, Levin MD. Macrocytic anaemia: causes and prognosis. Presented at: 26th annual meeting of the Dutch society for internal medicine; 2014 April 23-25; Maastricht, Netherlands
- **Stouten K,** Riedl JA, Levin MD. Macrocytic anaemia: causes and prognosis. Presented at: 8th Dutch Hematology Congress; 2014 January 22-24; Arnhem, Netherlands

Poster presentations:

- **Stouten K,** Riedl JA, Sonneveld P, Levin MD. Anaemia in general practice: the relevance of MCV. Presented at: 20th Congress of the European Hematology Association (EHA); 2015 June 11-14; Vienna, Austria
- **Stouten K,** Riedl JA, Sonneveld P, Levin MD. Anaemia in general practice. Presented at: 56th ASH annual meeting; 2014 December 6-9, San Francisco, CA, U.S.
- **Stouten K,** Riedl JA, Levin MD. Anaemia in general practice: causes and survival. Presented at: 19th Congress of the European Hematology Association (EHA); 2014 June 12-15, Milan, Italy

- **Stouten K,** Riedl JA, Levin MD. Macrocytic anaemia: causes and prognosis. Presented at: 55th ASH annual meeting; 2013 December 7-10, New Orleans, LA, U.S.

Teaching:

- Supervision Master student during internship
- Lecture ROIG
- Lecture Albert Event Clinical Chemistry

List of publications

2016

- **K. Stouten,** J.A. Riedl, R.J. van Houten, J. van Rosmalen, P. Sonneveld, M-D. Levin. Anaemia in general practice: an analysis of the relevance of the mean corpuscular volume when evaluating newly diagnosed anaemia. *Submitted for publication*
- **K. Stouten,** J.A. Riedl, R.J. van Houten, J. van Rosmalen, P. Sonneveld, M-D. Levin. The impact of anaemia aetiology on mortality in a large cohort of general practice patients. *Submitted for publication*
- **K. Stouten,** J.A. Riedl, J. Droogendijk, R. Castel, J. van Rosmalen, R.J. van Houten, P. Berendes, P. Sonneveld, M-D. Levin. Macrocytic anaemia in general practice: factors influencing diagnosis and prognosis. *Submitted for publication.*
- A. Schop, **K. Stouten,** J. Droogendijk, R.J. van Houten, J.A. Riedl, M-D. Levin. Adherence to the national guideline in patients with newly discovered anaemia of chronic disease in general practice. *Submitted for publication*
- A. Schop, **K. Stouten,** J. van Rosmalen, J. Droogendijk, R.J. van Houten, J.A. Riedl, M-D. Levin. The impact of adherence to the gold standard for diagnosis and treatment of iron deficiency anaemia on mortality in general practice. *Submitted for publication.*
- A. Egelé, **K.Stouten ,** L. van der Heul-Nieuwenhuijsen, L. de Bruin, R. Teuns, W. van Gelder, J.A. Riedl. Classification of several morphological red blood cell abnormalities by DM96 digital imaging. Int J Lab Haem. (2016) Jun; Epub ahead of print

2015

- **K. Stouten**, J.A. Riedl, M-D. Levin, W. van Gelder. Examination of peripheral blood smears: performance evaluation of a digital microscope system using a large-scale database Int J Lab Haem. (2015) Oct; 37(5) e137-140

- J.A. Riedl, **K. Stouten**, H. Ceelie, J. Boonstra, M-D. Levin, W. van Gelder. Interlaboratory reproducibility of blood morphology using the digital microscope. J Lab Autom. (2015) Dec; 20(6): 670-675

2014

- **K. Stouten,** M. van de Werken, I. Tchetverikov, M. Saboerali , H.J. Vermeer, R. Castel, F.M. Verheijen. Extreme elevation of serum angiotensin-converting enzyme (ACE) activity: always consider familial ACE hyperactivity. Ann Clin Biochem (2014) 51 (pt 2): 289-293

Acknowledgements

Onderzoek doe je absoluut niet alleen, ondanks dat het soms wel zo voelt. Dit proefschrift was er niet geweest zonder de volgende mensen, die ik op deze plek hartelijk wil bedanken.

Professor Sonneveld, hartelijk dank dat u mijn promotor wilde zijn. Ik heb uw kritische blik zeer gewaardeerd.

Mark-David, mijn co-promotor, zonder jouw inzet en enthousiasme waren PAGAS, de onderzoeksgroep en deze promotie er niet gekomen. Hartelijk dank voor al je toewijding en energie. De woorden 'sexy' en 'nog iets scherper maken' zijn overigens nog steeds verboden.

Jurgen, mijn andere co-promotor, je enthousiasme voor de digitale microscopie werkte zeer aanstekelijk en ik ben zeer blij dat de DM ook een onderdeel vormde van mijn promotietraject. Hartelijk dank voor al je energie en feedback.

De kleine commissie, professoren Boersma, Bindels en de Jonge, hartelijk dank voor het plaatsnemen in de commissie, en de tijd die u heeft vrijgemaakt om mijn proefschrift te beoordelen. Aan de overige leden van de grote commissie, hartelijk dank voor het lezen en beoordelen van mijn proefschrift.

Sten, Johan en Joost, hartelijk dank voor de statistische steun de afgelopen jaren.

Peter, hartelijk dank voor je feedback tijdens het onderzoeksoverleg op vooral mijn presentaties de afgelopen jaren.

Alle dames van de hematologie - Ria, Pleunie, Annemiek, Barbara, Carla, Linda, Peggy en Ria T-, hartelijk dank voor al jullie werk aan de digitale microscopie artikelen.

Marjan, mijn onderzoek bestond uit veel computerwerk maar af en toe kon ik toch niveau 3 induiken voor het betere pipetteerwerk. Dank je wel voor alle gezelligheid de afgelopen jaren.

Michael, Eric, Warry, François en Rob, hartelijk dank voor jullie interesse de afgelopen jaren. Dankzij de opleiding tot klinisch chemicus blijf ik ook de komende jaren nog in Dordt en ik hoop nog veel van jullie te leren.

Natuurlijk wil ik ook mijn kantoorgenootjes bedanken. Leonie, helaas hebben we weinig een kantoor gedeeld (al hebben we wel een tijdlang een bureau ‘gedeeld’). Ik wil je bedanken voor je steun en interesse de afgelopen jaren. Iris, je optimisme heeft me zeer geholpen tijdens het promotietraject. Ook jij zeer bedankt voor je steun en gezelligheid. Naura, je kwam misschien net op een verkeerd moment in mijn promotietraject bij ons te werken, want je hebt alle frustratie om het afmaken van het manuscript meegemaakt ;). Dank je voor je geduld en gezelligheid. Mirelle, je wist heel goed raad met mijn motivatieproblemen na weer een afwijzing en mijn besluiteloosheid over wanneer het

proefschrift nou eigenlijk echt af was. Waarschijnlijk lag het manuscript nog steeds op mijn bureau zonder jouw duwtjes in de rug. Hartelijk dank voor je geduld en steun.

Vooral de eerste maanden was deze promotie vooral solo-werk. Maar gelukkig wisten steeds meer mensen de onderzoeksruimte (a.k.a. kenniscentrum) op de 4e verdieping te vinden. Dankzij deze extra mensen werd het onderzoek ook gelijk een stuk leuker.

Jolanda, je hebt me geholpen met de eerste stapjes in de wondere wereld van PAGAS. Hartelijk dank!

Crista, voor je onderzoek verbleef je een half jaar op de 4e. Hartelijk dank voor het delen van je ervaring en je steun, beide hebben me zeer geholpen. Anne-Wil, dankzij je opleiding bivakkeerde jij ook een tijdje op de 4e. Ik blijf onder de indruk van je doorzettingsvermogen en hoe je alles combineert. Hartelijk dank voor je gezelligheid.

Annemarie, minion! Wat heb jij een enorme hoeveelheid werk gedaan voor de IDA en ACD stukken. En een flink deel ook nog eens naast je opleiding geneeskunde. Dank je wel voor al je inzet en gezelligheid.

Inge, onderzoeksmaatje! Altijd optimistisch en positief ingesteld. Ik denk niet dat er nog iemand is die zo blij kan worden van een gelukte SPSS analyse. Wanneer vieren we je eerste Blood-artikel? Bedankt voor alle steun en theeleut momentjes.

Hanah, mijn eerste onderzoeksmaatje! En alsof het onderzoek nog niet uitdagend genoeg was, besloten we ook nog 'even' de Nijmeegse Vierdaagse te lopen. Die hebben we gehaald en ook de promotie gaat lukken. Volgend jaar vieren we jouw promotie. Dank je wel voor alle gezelligheid en steun.

Om onderzoek te doen slagen, is af en toe ook ontspanning nodig. Al ging het in mijn vrije tijd ook regelmatig over de leuke en minder leuke kanten van de promotie.

Andre, dank je voor alle steun. Hopelijk heb ik vanaf nu wat meer tijd voor bordspellen. Colin, thank you for your boundless optimism and support.

Lenie, Fred, Danitsja, Michel, Marjolijn, Desiree, Tessa, Anne-Sophie, Justin, Benjamin, Annika en alle overige leden van Care4You door de jaren heen. Dank jullie wel voor alle lol de afgelopen jaren. Het was altijd een goede ontspanning naast het onderzoek.

Carmen, wat ben ik blij dat jij ooit besloot om bij BMW vakken te gaan volgen! Bedankt voor alle steun en goede raad. Je blijft optimistisch en weet me altijd weer de goede richting op te krijgen, binnen en buiten het onderzoek.

Becky, without your tireless editing, the English of my articles would still be abominable (thesaurus :P).You always made sure to remind me I could and would finish this thesis and

were always ready to join me in some ranting about the less fun aspects of a PhD project. Thank you for all your support and for being my paranimf.

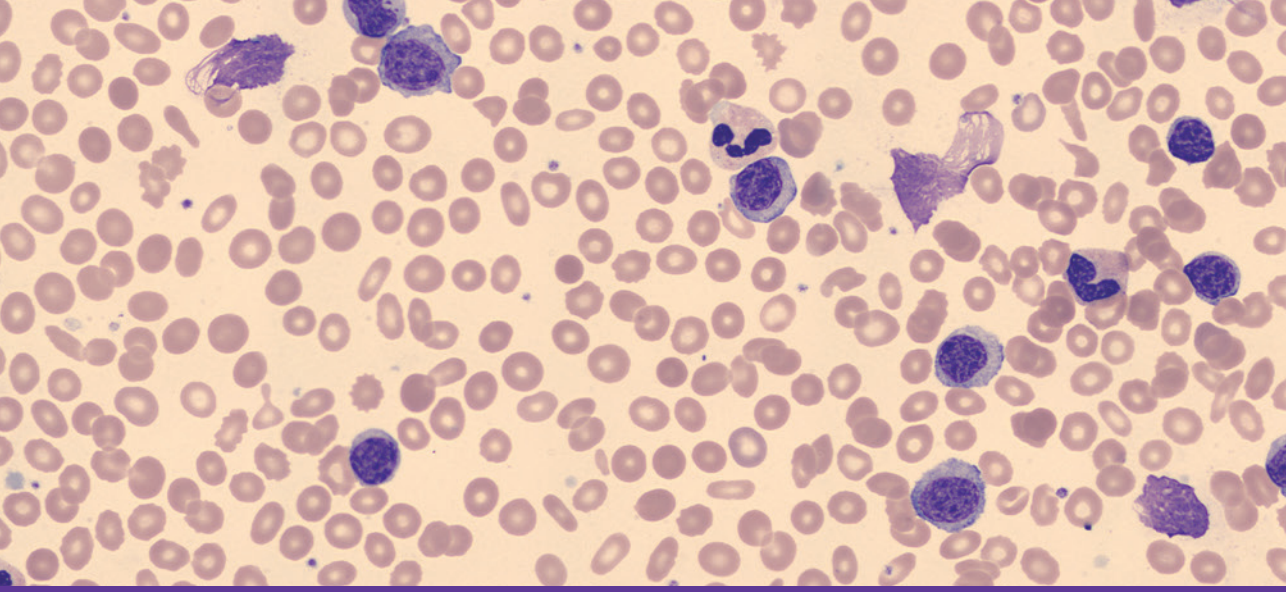
Kris, broertje! Ondanks dat we elkaar weinig zien, weet ik dat ik altijd op je kan rekenen. Dank je wel dat je mijn paranimf wilt zijn en veel geluk met Nadia in Amsterdam.

Mam en pap, dank jullie wel voor alle steun, geduld en liefde. Ik heb altijd alle vrijheid gehad en steun gekregen om zelf te kiezen welke richting ik op wilde. Het heeft me gebracht waar ik nu ben.

Curriculum Vitae

Karlijn Stouten was born on January 30th, 1987 in Deventer, the Netherlands. She grew up and attended primary school in the village of Heteren. In 1998 she started secondary school at Stedelijk Gymnasium Arnhem, and after a move, graduated in 2004 at Stedelijk Gymnasium 's-Hertogenbosch. That same year she began the bachelor Biomedical Sciences at the University of Utrecht, graduating in 2007. In 2008 she started the Master Drug Innovation, again in Utrecht, graduating in 2010. This was followed by the Master Forensic Archaeology and Crime Scene Investigation at the University of Bradford in the United Kingdom. In 2013 she began work on her PhD project at the department of Clinical Chemistry and Internal Medicine at the Albert Schweitzer Hospital, studying anaemia and its causes in general practice and contributing to the validation of digital microscopy systems, under the supervision of Prof. dr. Pieter Sonneveld, dr. Mark-David Levin and dr. Jurgen Riedl. The results derived from this research are described in this thesis. Since March 2016 she has been working as clinical chemist in training at the Albert Schweitzer Hospital in Dordrecht, with dr. F.M. Verheijen as supervisor and dr. M.A. Fouraux as co-supervisor.

Karlijn Stouten werd geboren op 30 januari 1987 in Deventer, Nederland. Ze groeide op in Heteren en ging hier naar de basisschool. In 1998 begon ze haar middelbare schooltijd op het Stedelijk Gymnasium Arnhem. Na een verhuizing behaalde zij in 2004 haar diploma aan het Stedelijk Gymnasium 's-Hertogenbosch. In datzelfde jaar begon ze met haar bachelor Biomedische Wetenschappen aan de Universiteit van Utrecht, welke in 2007 werd afgerond. In 2008 hervatte ze haar studie met de master Drug Innovation, opnieuw in Utrecht. Deze opleiding werd in 2010 afgerond waarna zij begon met de master Forensic Archaeology and Crime Scene Investigation aan de Universiteit van Bradford in het Verenigd Koninkrijk. In 2013 werd begonnen met het promotie onderzoek op de afdelingen klinische chemie en interne geneeskunde van het Albert Schweitzer ziekenhuis, met als onderwerpen anemie en de oorzaken van anemie in de huisartsenpraktijk en de validatie van digitale microscopie systemen, onder de supervisie van Prof. dr. Pieter Sonneveld, dr. Mark-David Levin en dr. Jurgen Riedl. De resultaten afkomstig van dit onderzoek worden beschreven in dit proefschrift. Vanaf maart 2016 is zij werkzaam als klinisch chemicus in opleiding in het Albert Schweitzer ziekenhuis in Dordrecht, met als opleider dr. F.M. Verheijen en plaatsvervangend opleider M.A. Fouraux.



It's a dangerous business, Frodo, going out of your door. You step into the Road , and if you don't keep your feet, there is no knowing where you might be swept off to.

J.R.R. Tolkien