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Cyclical Neutropenia: Data Analysis and Modeling Study

Caroline Haurie
Department of Physiology
McGill University, Montreal
February 1999

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements of the degree of Master in Science

Abstract

We review the salient clinical and laboratory features of cyclical neutropenia (CN) and other periodic hematological disorders, and the insight into these diseases afforded by mathematical modeling. Using Lomb periodogram analysis, we show that occurrence of significant cycling in the serial blood counts of neutropenic patients is very prevalent and the dynamics of all the cell lineages in CN can be modified by the administration of recombinant granulocyte colony stimulating factor (G-CSF). The analysis of the serial blood counts in the animal model of CN - the grey collie (GC), reveals a complex pattern of oscillations that we could reproduce with a model of hematopoiesis combining a peripheral control of granulopoiesis through G-CSF, together with an oscillatory input from the pluripotential stem cell. Both the human and GC data analysis suggests that CN results from a complex interaction between the stem cells, the mature cells and G-CSF, and that the regulation of the different blood cell lineages are strongly linked together.

Résumé

Nous faisons un compte rendu des caractéristiques cliniques et expérimentales de la neutropénie cyclique (NC) et des autres troubles hématologiques périodiques, ainsi que des modèles mathématiques qui éclairent l'étude de ces maladies. Grâce à l'utilisation du périodogramme de Lomb, nous mettons en évidence l'existence fréquente de périodicités significatives dans les énumérations sanguines des patients neutropéniques. Dans l'un ou l'autre des types de cellules sanguines, ces oscillations peuvent être modifiées par l'administration du granulocyte colony stimulating factor (G-CSF) recombinant. L'analyse statistique des énumérations sanguines des chiens grey collie (GC)–modèle animal de la NC, révèle un schéma complexe d'oscillations que nous pouvons reproduire par un modèle d'hématopoièse combinant le control périphérique de la granulopoièse par le G-CSF, et des oscillations périodiques émergeant du compartiment des cellules souches. Tant l'analyse des données humaines que celle des chiens GC suggèrent qu'une interaction complexe entre les cellules souches, les cellules différenciées, et la concentration de G-CSF, est à l'origine de la NC, et que les régulations des différentes lignées cellulaires sanguines sont étroitement liées entre elles.

Acknowledgements

The completion of this thesis could not have been done without the support of my supervisor Michael C. Mackey. I have greatly benefited from his real and rare intellectual honesty, and his availability for discussions on all matters.

I am grateful to him for giving so much freedom in my work. His patience, and friendly advising have made my beginnings in research a very enjoyable and enriching experience.

This work was possible only with D.C. Dale's cooperation. I am very grateful to him for sharing his expertise in hematology with us.

Thanks to all the staff and students from the CND for welcoming me in a very friendly fashion, especially Danny Kaplan, Leon Glass and Michael Guevara who shared part of their knowledge with me, and Linda Tracey and Frances Ezzy-Jorgensen who have helped me patiently to go through all the administration's labyrinths.

Contributions of Authors

Chapter 2 has been published in Blood (Vol. 92, p. 2629–2640, 1998). We would like to thank M. Pascal Fortin for digitizing the published data.

Chapter 3 has been published in Experimental Hematology (Vol. 27, p. 401–409, 1999). Data from neutropenic patients were collected by DC Dale from a Phase III clinical trial of G-CSF involving a number of investigators: Mary Ann Bonilla, Mark W. Davis, Arline M. Nakanishi, William P. Hammond, Joanne Kurtzberg, Winfred Wang, Ann Jakubowski, Elliott Winton, Parviz Lalezari, William Robinson, John A. Glaspy, Steve Emerson, Janice Gabrilove, Martha Vincent and Laurence A. Boxer.

Chapter 4 has been accepted for publication in Experimental Hematology (1999, in press). Grey collie dogs were maintained by D.C. Dale, blood cell counts were performed by R. Person in D.C. Dale's laboratory, and data analysis and modeling was by C. Haurie.

All data analysis was done by C. Haurie as was the bulk of the writing.

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Chapter 1

Introduction

The peculiar features of periodic hematological disorders are challenging our current conception of the regulation of hematopoiesis. These disorders affect the turnover of one or more of the blood cell lineages so that the density of mature cells in the blood oscillates with a relatively fixed period. As reviewed in Chapter 2, our current knowledge of the biology of hematopoiesis does not allow us to explain the occurrence of most of the periodic hematological disorders. The fact that these are not constant, but rather dynamical [52], disorders gives us a means to study the functioning in time of the regulatory mechanisms that drive hematopoiesis and that normally maintain functional densities of cells in the blood. They compel us to construct models of the regulatory network that controls hematopoiesis and to look for principles that determine the particular features of the diseases states, such as the period, amplitude and phase relations of the oscillations.

Among these disorders, cyclical neutropenia (CN) affects several cell lineages in different ways, which makes it both more difficult to understand

and more interesting to study. Its particular features raise such fundamental questions as: How are the mechanisms regulating the production of the different blood cell lineages related? To what extent does the existence of a common ancestor (the pluripotential stem cell) imply an interdependence of the regulation of the different blood cell lineages? How independent are the early stem cells from the fully differentiated, mature cells?

Whereas CN probably involves regulatory loops acting on early stem cells in the bone marrow, there are very few studies of the bone marrow counts in CN patients because of the technical difficulty of such studies. Serial peripheral blood counts, which are much easier to collect, may however provide precious information on this disease.

We did an extensive statistical analysis of serial blood counts from cyclical neutropenic patients and grey collie (GC) dogs, which are an animal model of CN, as well as serial blood counts from neutropenic patients. We found several features in the dynamics of CN in both humans and dogs that had not been observed previously and that allowed us to compare the validity of several models of hematopoiesis. These models and the periodic hematological disorders are reviewed in Chapter 2. Chapter 3 and 4 summarize the results of the analysis of the blood counts in neutropenic patients and grey collie dogs respectively.

Chapter 2

Cyclical Neutropenia and Other Periodic Hematological Disorders: A Review of Mechanisms and Mathematical Models

2.1 Regulation of Hematopoiesis

Mature blood cells and recognizable precursors in the bone marrow ultimately derive from a small population of morphologically undifferentiated cells, the hemopoietic stem cells (HSC), which have a high proliferative potential and sustain hematopoiesis throughout life (Figure 2.1). The earliest HSC are totipotent and have a high self-renewal capacity [2, 8, 98]. These qualities are progressively lost as the stem cells differentiate. Their progeny, the progenitor cells, or colony forming units (CFUs), are committed to one cell lineage. They proliferate and mature to form large colonies of erythro-

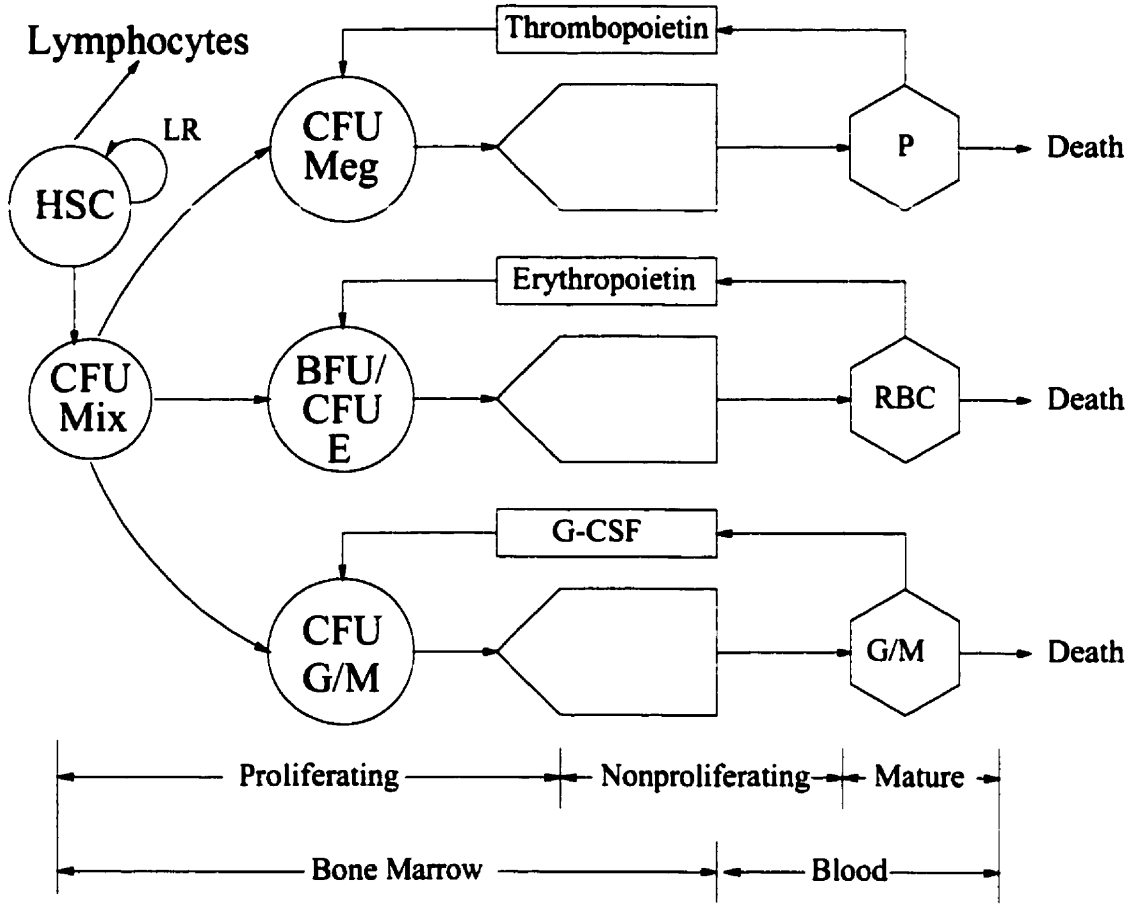

Figure 2.1: The architecture and control of hematopoiesis. This figure gives a schematic representation of the architecture and control of platelet (P), red blood cell (RBC), and monocyte (M) and granulocyte (G) (including neutrophil, basophil and eosinophil) production. Various presumptive control loops mediated by thrombopoietin (TPO), erythropoietin (EPO), and the granulocyte colony stimulating factor (G-CSF) are indicated, as well as a local regulatory (LR) loop within the totipotent hematopoietic stem cell (HSC) population. CFU (BFU) refers to the various colony (burst) forming units (Meg = megakaryocyte, Mix = mixed, E = erythroid, and G/M = granulocyte/monocyte) which are the *in vitro* analogs of the *in vivo* committed stem cells (CSC).

cytes, granulocytes, monocytes or megakaryocytes. The growth of CFUs *in vitro* depends on lineage-specific growth factors, such as erythropoietin (EPO), thrombopoietin (TPO), and the granulocyte, monocyte and granulocyte/monocyte colony stimulating factors (G-CSF, M-CSF and GM-CSF).

EPO adjusts erythropoiesis to the demand for O_2 in the body. A fall in tissue pO_2 levels (in response to any one of a number of factors) leads to an increase in the renal production of EPO. This in turn leads to an increased cellular production by the primitive erythroid precursors (CFU-E) and, ultimately, to an increase in the erythrocyte mass and hence the tissue pO_2 levels. This increased cellular production triggered by EPO is due, at least in part, to an inhibition of pre-programmed cell death (apoptosis) [3, 183] in the CFU-E and their immediate progeny. Thus, EPO mediates a negative feedback such that an increase (decrease) in the erythrocyte mass leads to a decrease (increase) in erythrocyte production.

The mechanisms regulating granulopoiesis are not as well understood. G-CSF, the primary controlling agent of granulopoiesis, is a completely sequenced high molecular weight molecule [123] produced by a number of tissues (fibroblasts, endothelial, epithelial) and circulating cells (monocytes). G-CSF is absolutely essential for the growth of the granulocytic progenitor cells CFU-G *in vitro* [208]. CFU-G colony growth is a sigmoidally increasing function of increasing G-CSF concentration [66, 6]. One of the modes of action of G-CSF, along with several other cytokines, is to decrease apoptosis [91, 152, 208, 209]. Additionally there is a clear shortening of the neutrophil

maturation time under the action of G-CSF [157].

The important role of G-CSF for the *in vivo* control of granulopoiesis was demonstrated by Lieschke *et al.* [101]. They showed that mice lacking G-CSF (due to an ablation of the G-CSF gene in embryonal stem cells) have pronounced neutropenia and reduction of the marrow granulocyte precursor cells by a factor of 50%. The administration of exogenous G-CSF corrects the neutropenia in one day and restores the marrow composition to that typical of a normal wild type mouse within 4 days. G-CSF also rapidly corrects neutropenia of diverse causes in humans [18, 96, 97, 105, 106, 155] and other mammals [23, 107, 127].

Several studies have shown an inverse relation between circulating neutrophil density and serum levels of G-CSF [87, 122, 188, 196]. Coupled with the *in vivo* dependency of granulopoiesis on G-CSF, this inverse relationship suggests that the neutrophils would regulate their own production through a negative feedback, as is the case with erythrocytes, where an increase (decrease) in the number of circulating neutrophils would induce a decrease (increase) in the production of neutrophils through the adjustement of G-CSF levels. Although mature neutrophils bear receptors for G-CSF and for GM-CSF the role of these receptors in governing neutrophil production is not yet known.

The regulation of thrombopoiesis presumably involves similar negative feedback loops. Megakaryopoiesis can be separated in two processes: the proliferation and differentiation of megakaryocytic progenitor cells, and the

complex process of maturation of precursor cells which includes a variable number of endomitotic nuclear divisions, cytoplasmic growth and maturation, and the development of platelet-specific structures.

Until recently, the regulation of megakaryopoiesis was thought to include two separated control mechanisms [76, 120]: First, a regulatory loop mediated by a Meg-CSA responding to megakaryocyte demand and acting on the proliferation of CFU-Meg; Second, a thrombocytopenia-activated control of the maturation of megakaryocytes, mediated by thrombopoietin (TPO). Several growth factors, such as Il-11, Il-6, Il-3 and GM-CSF possess either one (but not both) of these activities and promote platelet production *in vivo*. However, none was found to be specific to the megakaryocytic lineage [76].

A lineage-specific factor which is the ligand for Mpl receptor has been cloned recently, which has both Meg-CSA and TPO activity [199]. Plasma TPO levels are increased in thrombocytopenic patients [146]. Administration of TPO to non-human primates induces up to six- to seven- fold increases in the platelet counts [85, 146]. It is now thought that the Mpl ligand mediates a negative feedback loop regulating platelet production [180].

There are more than 15 other cytokines acting on hematopoiesis [123], with broad, redundant actions [123, 170]. In vitro studies have shown that Il-3 and Stem Cell Factor (SCF, Kit ligand) are involved with the survival of HSC, while Il-6, Il-11, Il-12 and G-CSF have synergistic effects on the entry into cycling of dormant HSC [97, 148]. In contrast to the committed progenitors, the growth of HSC *in vitro* thus depends on the interaction

of several cytokines. The action of G-CSF on the cycling of HSC in vitro is supported by in vivo effects. While suppression of G-CSF only affects granulopoiesis [101], G-CSF administration can result in multilineage recovery [186] and modify the kinetics of colony forming unit (spleen) (CFU-S) [126]. Little is known about how the self-maintenance of the HSC population is achieved. HSC are usually in a dormant state but are triggered to proliferate after transplantation into irradiated hosts [140]. The specific mechanisms regulating the differentiation commitment of HSC are unknown [147]. Self-maintainance of HSC depends on the balance between self-renewal and differentiation. Mechanisms which could support auto-regulatory feedback control loops controlling HSC kinetics are starting to be investigated [144].

2.2 Periodic Hematological Disorders

2.2.1 Cyclical Neutropenia (CN)

General Features

Cyclical neutropenia (CN) has been the most extensively studied periodic hematological disorder. Its hallmark is a periodic fall in the circulating neutrophil numbers from normal values to very low values. In humans it occurs sporadically or as an autosomal dominantly inherited disorder, and the period is typically reported to fall in the range of 19–21 days [27] though recent data indicate that longer periods occur in some patients [71]. Our understanding of CN has been greatly aided by the discovery that the grey collie

suffers from a very similar disease. The canine disorder closely resembles human CN with the exception of the period which ranges from 11 to 15 days [72] and the maximum neutrophil counts which are higher than for humans. For reviews see [27, 29, 82, 94, 95, 151, 158, 210].

It is now clear that in both human CN [28, 25, 71, 75] and the grey collie [61, 72] there is not only a periodic fall in the circulating neutrophil levels, but also a corresponding oscillation of platelets, often the monocytes and eosinophils, and occasionally the reticulocytes and lymphocytes (see Figure 2.2a). The monocyte, eosinophil, platelet, and reticulocyte oscillations are generally from normal to high levels, in contrast to the neutrophils which oscillate from near normal to extremely low levels. Often (but not always) the period of the oscillation in these other cell lines is the same as the period in the neutrophils.

The clinical criteria for a diagnosis of CN have varied widely, though the criteria used by Dale *et al.* [33] are widely accepted. These are that a patient must display an absolute neutrophil count (ANC) less than 0.5×10^9/L on at least three to five consecutive days per cycle for each of three regularly spaced cycles. Usually this requires blood cell counts three times per week for six to eight weeks. Most patients will also have significant symptoms (malaise, anorexia) and signs (e.g. fever, mouth ulcers, lymphoadenopathy) during their neutropenic periods. Sometimes it is difficult to make the diagnosis with certainty. Using periodogram analysis, some patients classified as having CN do not in fact display any significant periodicity while other patients classified

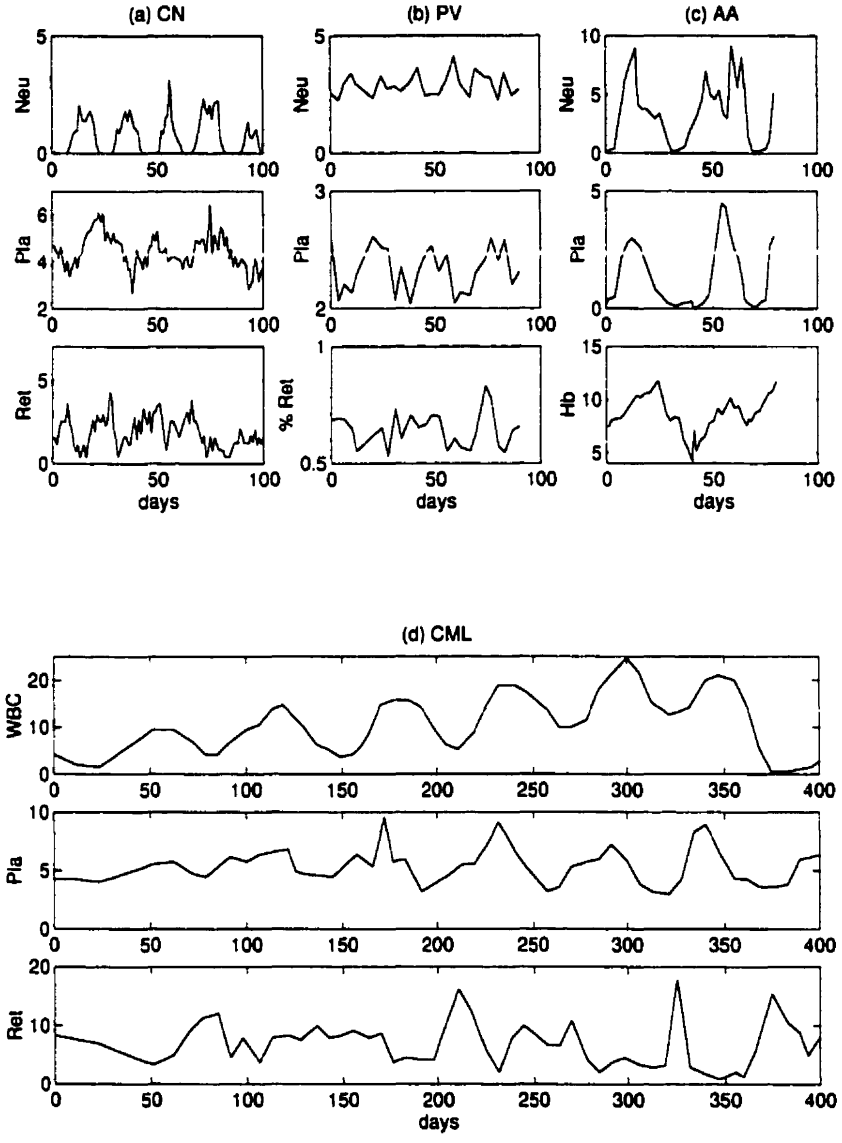

Figure 2.2: Representative patterns of circulating cell levels in four periodic hematological disorders considered in this review. Part (a) illustrates cyclical neutropenia (CN) [61], (b) polycythemia vera (PV) [130], (c) aplastic anemia (AA) [133], and (d) periodic chronic myelogenous leukemia (CML) [20]. The density scales are: Neutrophils, 10^3 cells/mm^3; white blood cells, 10^4 cells/mm^3; platelets, 10^5 cells/mm^3; reticulocytes, 10^4 cells/mm^3; and Hb, g/dl.

with either congenital or idiopathic neutropenia do display significant cycling [71].

Origin

Transplantation studies show that the origin of the defect in CN is resident in one of the stem cell populations of the bone marrow [26, 83, 84, 197, 92, 153]. Studies of bone marrow cellularity throughout a complete cycle in humans with CN show that there is an orderly cell density wave that proceeds successively through the myeloblasts, promyelocytes, and myelocytes and then enters the maturation compartment before being manifested in the circulation [16, 61]. Further studies have shown that this wave extends back into the CFU-G [79], CFU-E [39, 38, 67, 81] as well as in the BFU-E and CFU-GM [1, 67], suggesting that it may originate in the totipotent HSC populations.

Studies in the grey collie [6, 108] and in humans [66, 212] show that the responsiveness of granulocyte committed progenitor cells to G-CSF is greatly attenuated in CN compared to normal. Patients also differ from normal in their requirements for GM-CSF but not for IL-3 [212].

In CN the levels of colony stimulating activity (CSA - related to G-CSF) fluctuate inversely with the circulating neutrophil levels and in phase with the peak in monocyte numbers [30, 60, 129]. EPO levels oscillate approximately in phase with the reticulocyte oscillation [60]. Dunn *et al.* [40] have also shown a periodic stimulation/repression of CFU-S (the HSC) by conditioned media derived from cyclic neutropenic marrow. It is unclear if these

correlations and inverse correlations between levels of circulating cells and putative humoral regulators are related to the cause of CN, or are simply a secondary manifestation of some other defect.

Effect of Phlebotomy and Hypertransfusion

The effect of bleeding and/or hypertransfusion on the hematological status of grey collies gives interesting results [4]. In the untreated grey collie EPO levels cycle out of phase with the reticuloctyes and virtually in phase with the neutrophil counts. After phlebotomy (bleeding of between 10% and 20% of the blood volume) the cycles in the neutrophils and reticulocytes continue as before the procedure, and there is no change in the relative phase between the cycles of the two cell types. Hypertransfusion (with homologous red cells) completely eliminates the reticulocyte cycling (as long as the hematocrit remained elevated), but has no discernible effect on the neutrophil cycle. Most significantly, when the hematocrit falls back to normal levels and the reticulocyte cycle returns, the phase relation between the neutrophils and the reticulocytes is the same as before the hypertransfusion. These observations suggest that the source of the oscillations in CN is relatively insensitive to any feedback regulators involved in peripheral neutrophil and erythrocyte control, whose levels would be modified with the alteration of the density of circulating cells, and is consistent with a relatively autonomous oscillation in the HSC (c.f. Section 2.3.2).

Effect of Cytokine and Lithium Therapy

In both the grey collie [65, 108] and in humans with CN [68, 124, 211] administration of G-CSF leads to an increase in the mean value of the peripheral neutrophil counts by a factor of as much as 10 to 20, associated with a clear improvement of the clinical symptoms. However G-CSF does not obliterate the cycling in humans, but rather induces an increase in the amplitude of the oscillations and a decrease in the period of the oscillations in all the cell lineages, from 21 to 14 days [68]. In human and canine CN, GM-CSF leads to an increase in neutrophil count by a factor of between 1.5 and 3.5, which is much less than achieved by G-CSF. In one report, GM-CSF obliterated cycling [211]. Although recombinant canine stem cell factor (rc-SCF) does not cause neutrophilia in grey collies, it does obliterate the oscillations of CN. Lithium therapy in grey collies [67, 69] has uniformly yielded an elimination of the severe neutropenic phases, and a diminution in the amplitude of the oscillations without any apparent change in the period of the oscillation. In humans, there are variable results with lithium [177, 192] and the largest study showed lack of efficacy and toxicity problems [64].

2.2.2 Other Periodic Hematological Disorders Associated with Bone Marrow Defects

Periodic CML

Chronic myelogenous leukemia (CML) is a hematopoietic stem cell disease characterized by granulocytosis and splenomegaly [57]. In 90 percent of the

cases, the hematopoietic cells contain a translocation between chromosomes 9 and 22 which leads to the shortening of chromosome 22, referred to as the Philadelphia (Ph) chromosome. The disease is acquired and results from the malignant transformation of a single pluripotential stem cell, as shown by the presence of a single G-6PD isoenzyme in the red cells, neutrophils, eosinophils, basophils, monocytes and platelets in women with CML who are heterozygotes for isoenzymes A and B [44]. The leukocyte count is greater than 100×10^9/L in 50 percent of the cases and it rises progressively in untreated patients. The platelet and reticulocyte counts can also be mildly elevated. In most cases, the disease eventually develops into acute leukemia.

Morley was the first to describe oscillations in the leukocyte count of CML patients in 1967 [136]. Several other cases of cyclic leukocytosis in CML have now been reported [20, 35, 47, 48, 77, 78, 88, 118, 117, 121, 149, 161, 179, 190, 193, 214]. The leukocyte count usually cycles with an amplitude of 30 to 200×10^9 cells/L and with periods ranging from approximately 30 to 100 days. Oscillations of other blood elements in association with CML have been observed. The platelets and sometimes the reticulocytes then oscillate with the same period as the leukocytes, around normal or elevated numbers. There have been no specific studies of hematopoiesis in patients with periodic CML. There is also a report of one patient with periodic acute myelogenous leukemia [117].

Polycythemia Vera and Aplastic Anemia

Polycythemia vera is characterized by an increased and uncontrolled proliferation of all the hematopoietic progenitors and it involves, like CML, the transformation of a single pluripotential stem cell. Two patients with polycythemia vera (PV) were reported with cycling of the reticulocyte, platelet and neutrophil counts in one case (cf. Figure 2.2b), and cycling only of the reticulocyte counts in the other. The period of the oscillations was 27 days in the platelets, 15 days in the neutrophils and 17 days in the reticulocytes [130].

Finally, clear oscillations in the platelet, reticulocyte and neutrophil counts (cf. Figure 2.2c) were reported in a patient diagnosed as having aplastic anemia (AA) [133] and in a patient with pancytopenia [12], with periods of 40 and 100 days respectively.

Cytokine Induced Cycling

G-CSF is routinely used in a variety of clinical settings, for example to treat chronic neutropenia or to accelerate recovery from bone marrow transplant and/or chemotherapy [33]. G-CSF may induce oscillations in the level of circulating neutrophils of neutropenic individuals [15, 71, 80, 198]. When these oscillations arise they always seem to be of relatively low period (on the order of 7 to 15 days), and their origin is unclear. There has also been one report of GM-CSF induced 40 day cycling in a patient with CML following bone marrow transplant [53].

Induction of Cycling by Chemotherapy or Radiation

Several reports describe induction of a CN-like condition by the chemotherapeutic agent cyclophosphamide. In mongrel dogs on cyclophosphamide the observed period was on the order of 11 to 17 days, depending on the dose of cyclophosphamide [134, 135]. In a human undergoing cyclophosphamide treatment, cycling with a period of 5.7 days was reported [32]. Gidáli et al [51] observed oscillations in the granulocyte count and the reticulocyte counts with three weeks periodicity in mice after mild irradiation. They observed an overshooting regeneration in the reticulocytes and the thrombocytes but not in the granulocytes. While the CFU-S returned to normal levels rapidly, the proliferation rate of CFU-S stayed abnormally elevated.

Five CML patients receiving hydroxyurea showed oscillations in their neutrophils, monocytes, platelets and reticulocytes with periods in the range of 30 to 50 days [88]. In one patient an increase of the hydroxurea dose led to a cessation of the oscillations. Chikkappa *et al.* [21] report a CN like condition (period between 15 and 25 days) in a patient with multiple myeloma after three years of chemotherapy.

A ^{89}Sr–induced cyclic erythropoiesis has been described in two congenitally anemic strains of mice, W/W^v and S1/S1^d [50, 49, 62]. W/W^v mice suffer from a defect in the HSC and in S1/S1^d mice the hematopoietic microenvironment is defective.

The induction of cycling by ^{89}S can be understood as a response to elevated cell death (c.f. Section 2.3.2), as can the dynamic effects of chemother-

apy.

2.2.3 Periodic Hematological Disorders of Peripheral Origin: AIHA and Cyclical Thrombocytopoiesis

Periodic auto-immune hemolytic anemia (AIHA) is a rare form of haemolytic anemia in humans [159]. Periodic AIHA, with a period of 16 to 17 days in hemoglobin and reticulocyte counts, has been induced in rabbits by using red blood cell auto-antibodies [150].

Cyclic thrombocytopenia, in which platelet counts oscillate from normal to very low values, has been observed with periods between 20 and 40 days [5, 7, 11, 17, 19, 22, 24, 34, 36, 42, 54, 100, 184, 189, 195, 207, 215] and reviewed by Cohen and Cooney [24]. Though it has been claimed that oscillations could be detected in the platelet counts of normal individuals with the same range of periods [131, 194], this conclusion may not be statistically justified.

2.3 Hypotheses for the Origin of Periodic Hematopoiesis

In clinical reports of periodic diseases affecting hematopoiesis, oscillations have usually been observed only in the blood counts without examinations of bone marrow precursors and progenitor cells. However, even in the case of CN where the kinetics of hematopoiesis have been extensively studied, the mechanisms responsible for the onset of periodic oscillations are still unknown. A number of mathematical models have been put forward which

suggest possible mechanisms for the origin of oscillations in hematopoiesis. These models fall into two major categories: The first group identifies the origin of the oscillations with the loss of stability in peripheral control loops adjusting the production rate of blood precursors to the number of mature cells in the blood and mediated by TPO, EPO, and G-CSF (cf. Figure 2.1). The second group is based on the assumption that oscillations arise in stem cell populations as a consequence of the loss of stability of auto-regulatory (local, LR) control loops (cf. Figure 2.1). A few authors have also modeled interactions between these two types of control loops. (See Dunn [37] and Fisher [45] for reviews.)

2.3.1 Models of the Peripheral Control of Hematopoiesis

It is well known that simple negative feedback systems, like the erythroid control system, have a tendency to oscillate. The relative detail known about erythrocyte production control has not escaped the attention of modelers who have mathematically explored ways to explain the results of laboratory manipulations in rodents [74, 102, 103, 191, 205, 213, 217], rabbits [90, 150], and the nature of the human erythropoietic regulatory system in health and disease [93, 128, 137, 206].

The control of erythropoiesis by EPO can be modeled by a single delayed differential equation describing the rate of change of RBC production as a function of the death rate of circulating erythrocytes, the rate of change of cell production following a given perturbation of the circulating RBC num-

ber, and the maturation time of erythrocyte progenitors. The transition from damped to stable oscillations, which characterizes the onset of periodic hematopoiesis, depends on the modification of one or several of these controlling parameters. In AIHA the death rate of circulating RBC is increased, while the other parameters lie within normal ranges. The mathematical modeling of the control of RBC production by EPO indicates that such an increase in the destruction rate of circulating erythrocytes will induce periodic fluctuations of erythropoiesis around a low average, with periods similar to the ones observed in AIHA [10, 111, 115]. From a modeling perspective, the laboratory version of rabbit AIHA is thus one of the best understood periodic haematological diseases.

Similar mathematical treatments have been applied to the control of granulopoiesis and megakaryopoiesis, even though the existence of functional peripheral feedback in these systems is still hypothetical.

A few authors have formulated models for the regulation of thrombopoiesis [41, 56, 63, 194, 203] assuming the existence of a negative feedback loop mediated by TPO. Bélair and Mackey [9] specifically considered cyclical thrombocytopenia. They speculated that elevations in the random destruction rate of platelets could give rise to the characteristic patterns observed in cyclical thrombocytopenia. Though modeling results based on this assumption yielded results qualitatively consistent with the clinical data, there is still much room for further study of this problem.

Several models of granulopoiesis incorporate a peripheral negative feed-

back loop [13, 14, 46, 165, 169, 166, 167, 168, 185, 187, 200, 204]. In the grey collie and in CN patients, the survival of circulating neutrophils is normal [28]. This implies that there is not a periodic elevation of the peripheral death rate of neutrophils in CN, but rather a periodic modulation of marrow cell production. An alteration of the peripheral control of granulopoesis has been proposed as the mechanism of CN by several authors [86, 89, 109, 134, 135, 132, 133, 160, 178, 182, 172, 173, 174, 175, 176, 202].

There is experimental evidence of alterations in the kinetics of granulopoiesis in CN, i.e. the modification of the distribution of maturation time and the subnormal responsiveness of granulocytic progenitors to CSF. However recent modeling indicates that these are insufficient to account for a destabilization of the putative peripheral feedback control of granulocytopoiesis [73]. Moreover, the lack of effect of hypertransfusion or phlebotomy on either cycle (neutrophil or reticulocyte) strongly implies that there is not a direct role of peripheral feedback loops in the origin of the cycling in CN.

A few attempts have also been made to model periodic CML based on the peripheral control of granulopoiesis [200, 201, 113] but these are also unsatisfactory in the sense that the models have assumptions on the kinetics of granulopoiesis in CML that are biologically unrealistic [154, 216].

The occurrence of oscillations in several blood elements in CN and CML strongly suggests that the oscillations are not a consequence of a lineage-specific regulatory loop but rather of regulatory mechanisms affecting all hematopoiesis. The existence of a peripheral feedback loop controlling gran-

ulopoiesis can thus not be supported by the occurrence of oscillations in CN and CML.

2.3.2 Models of the Autoregulatory Control of HSC

Except for AIHA and cyclical thrombocytopenia, the periodic hematological disorders that have been described are characterized by the occurrence of oscillations in many or all of the peripheral cellular elements (neutrophils, platelets, lymphocytes and reticulocytes). Many have speculated that the origin of these oscillations is in the common HSC population feeding progeny into the differentiated cell lines.

Mackey [110, 112] proposed that there could be a loss of stability in the stem cell population independent of feedback from peripheral circulating cell types. He analyzed a mathematical model for a stem cell population where the proportion of cells entering proliferation depends on the size of the population in G_0 (Figure 2.3). The efflux into the different committed cell lineages depends on the size of the population, which varies with the rate of proliferation and the cell death rate.

Other authors [55, 58, 59, 139, 138, 145, 162, 163, 164] have considered the dynamics of auto-regulatory stem cell populations from a modeling perspective applied to various experimental and clinical situations. Nečas *et al.* have developed such a modeling study based on experimental evidence that the number of stem cells entering proliferation is controlled by the number of DNA-synthesizing cells [141, 142, 143, 144]. Cell to cell interactions or

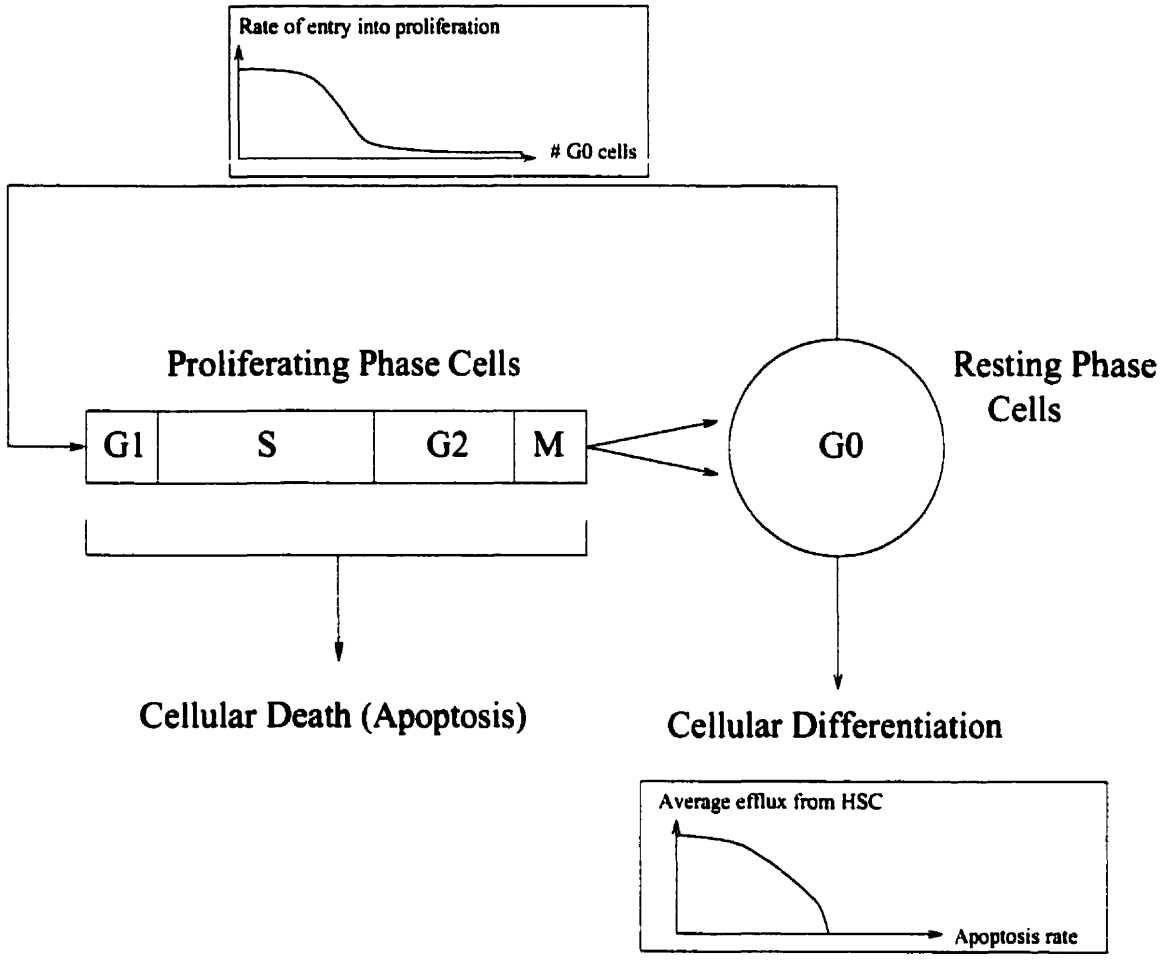

Figure 2.3: A schematic representation of the control of HSC regeneration. Proliferating phase cells include those cells in G_1, S (DNA synthesis), G_2, and M (mitosis) while the resting phase cells are in the G_0 phase. Local regulatory influences are exerted via a cell number dependent variation in the rate of entry into proliferation. Differentiation into all of the committed stem cell populations occurs from the G_0 population, while there is a loss of proliferating phase cells due to apoptosis. See [110, 125] for further details.

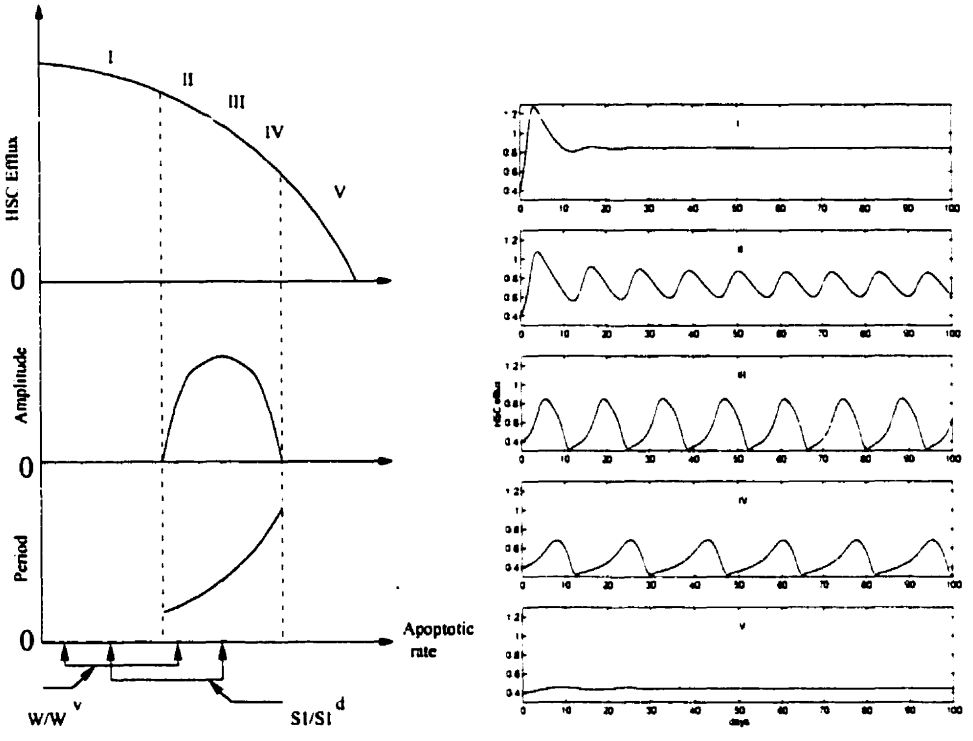

Figure 2.4: a) Schematic representation of the effects of increasing apoptotic rate on the HSC dynamics, as predicted from the model proposed in [110, 125]. The diagram shows the effect of administering the same dose of ^{89}Sr to W/W^{v} and Sl/Sl^{d} mice on the amplitude and the period of the oscillations. The dashed lines show the onset and the end of oscillations as the apoptotic rate increases. b) Computer simulations of the normalized HSC efflux predicted from [110] for increasing apoptotic rates (from I to V). As the apoptotic rate is increased, the period of the oscillations increases and the amplitude increases and decreases consecutively.

cytokines which are essential for the growth of HSC in vitro, such as Il-3 and SCF, could be involved in the autoregulation of HSC proliferation.

Because of the delay in the autoregulation loop, due to the time necessary for replicating cells to go through the S phase and the mitotic phase, this system has a tendency to oscillate. In normal conditions it is assumed that the population does not oscillate. Mackey showed that an abnormally large irreversible cell loss within the proliferating compartment, which can represent either apoptosis or other cell death, would induce oscillations of the number of stem cells [110]. Figure 2.4 shows the variations in the amplitude and the period of the oscillations predicted by the model as the apoptotic rate is increased. An increase in the apoptotic rate of HSC also induces a decrease in the average efflux from the HSC to all the cell lineages. This is consistent with the occurrence of oscillations in all the blood elements in some patients with AA, where all the blood cell counts are low, as well as the oscillations observed in several cell lineages after chemotherapy or radiation. The range of periods obtained by Mackey depends largely on the rate of irreversible cell loss, and on the cell cycle time delay. Depending on the value of these parameters, the period can vary from 16 to 43 days in humans and from 9 to 26 days in dogs. In most periodic hematological disorders, large differences consistent with these values have been observed in the period of the oscillations between different individuals.

The model also gives a plausible explanation for the ^{89}Sr induction of cyclic erythropoiesis in the two congenitally anemic strains of mice, W/W^v

and S1/S1^d (cf Figure 2.4). Assume that the difference between W/W^v and S1/S1^d mice is solely related to differences in the rate of apoptosis [116]. (The observation that S1/S1^d mice are more refractory to erythropoietin than W/W^v mice suggests that the apoptotic rate is higher in S1/S1^d.) The results of [110] predict that a higher rate of apoptosis would increase the likelihood that an oscillation in erythrocyte number occurs. Indeed, in contrast to W/W^v mice, $\sim$ 40 % of S1/S1^d mice have "spontaneous" oscillations in their hematocrit [49, 50]. In both strains of mice, a single dose of ^{89}Sr is sufficient to increase apoptosis into a range associated with oscillations in erythrocyte number. Since the apoptotic rate for the S1/S1^d mice is greater than that for W/W^v mice prior to ^{89}Sr, it is reasonable to expect that it will also be higher following administration of equal doses of ^{89}Sr to both strains of mice. As predicted, the period of the oscillation is longer, the amplitude is larger and the mean hematocrit is lower for S1/S1^d mice than they are for W/W^v mice [125].

2.3.3 The particular cases of CN and CML

Destabilization of an early HSC population may occur after radiation or chemotherapy, and in AA, and may result in oscillations with a large range of periods in all the blood elements. A similar destabilization may be at the origin of the oscillations observed in CN and CML. Modification of any of the parameters in the model described in [110] (Figure 2.3) can potentially induce the onset of oscillations.

Even though all the blood elements are often oscillating in periodic CML and CN, the defect in these two disorders primarily affects granulopoiesis. The abnormal responsiveness of CFU-G explains the low ANC levels in CN, which may induce an alteration in the cytokine levels. The fact that G-CSF is the only cytokine shown to alter the period of the oscillations in all the blood elements shows that it plays a crucial role in the mechanism at the origin of the oscillations in CN. The finding that G-CSF not only regulates granulopoiesis but also affects the kinetics of early HSC suggests that its effect on the oscillations in CN may be mediated through the modification of HSC dynamics. In vitro studies of the effect of G-CSF suggest that it increases the rate of entry into cycling, which is likely to destabilize the steady state while increasing the average size of the population. This is consistent with the mildly elevated platelet, monocyte and lymphocyte counts often observed in CN. Similar mechanisms may also induce the oscillations in CML.

The mechanisms underlying the complex dynamical features of CN and periodic CML will more likely be understood with the use of models that include both the local autoregulation of early stem cells and its relation with the more mature hemopoietic compartments. The multilevel effect of cytokines such as G-CSF suggests indeed that strong relationships exist between the regulation of early and late hemopoietic compartments.

2.4 Conclusion

This review focuses on the clinical and laboratory findings in periodic hematopoietic diseases including cyclical neutropenia, periodic chronic myelogenous leukemia, aplastic anemia, polycythemia vera, auto-immune hemolytic anemia, and cyclical thrombocytopenia. With the exception of the latter two, the available evidence indicates a broad involvement of the entire hematopoietic system since cycling is typically observed in more than one of the mature hematopoietic cell types.

Cycling in one or several hematopoietic cell lineages is probably much more frequent than reported, and would be detected if serial blood counts were systematically performed. These observations suggest a major derangement of the dynamics of one or more of the stem cell populations such that they become unstable and generate sustained oscillations that are manifested in more than one of their progenitor lines. Mathematical modeling studies suggest that there are several ways in which the HSC dynamics can be destabilized and give rise to oscillations if they are controlled by an autoregulatory loop. The finding that lineage-specific cytokines also have an effect on early HSC regulation implies that oscillations could arise as a result of the alteration of only one compartment, such as observed in CN and CML. Analysis of the effect of the cytokines on the dynamical features of these disorders, through modeling studies, may be a key to the understanding of the nature of early hematopoiesis regulation.

Chapter 3

Occurrence of Periodic Oscillations in the Differential Blood Counts of Congenital, Idiopathic and Cyclical Neutropenic Patients Before and During Treatment with G-CSF

3.1 Introduction

Severe chronic neutropenia (SCN) is a general term for a group of conditions characterized by a persistent absolute neutrophil count (ANC) in the blood lower than 0.5×10^9cells/L. The neutrophil half life in the circulation is usually normal, and the function of the cells is normal or only moderately impaired [157, 31]. These conditions are attributed to reduced or ineffective production of neutrophils by the bone marrow based on marrow morphology

and kinetic studies [157]. In many cases there is a dramatic reduction in the absolute number of neutrophil precursors in the bone marrow. The origin of this marrow depression is unknown.

SCN includes congenital neutropenia (ConN) or congenital agranulocytosis, which is usually recognized soon after birth; idiopathic neutropenia (IN), which is acquired during childhood or adulthood; and cyclical neutropenia (CN). Cyclical neutropenia has been described as regularly occurring episodes of severe neutropenia with a period of 3 weeks [27]. Approximately two-thirds of CN cases have a family history suggesting an autosomal dominant inheritance, but the disease can also occur as an acquired disorder. Grey collie dogs have cyclical neutropenia inherited as an autosomal recessive disorder. In the grey collie, the monocytes, eosinophils, platelets, and reticulocytes oscillate with the same period as the neutrophils but around or above normal values [29, 95]. In humans with CN the platelets, often the monocytes and eosinophils, and occasionally the reticulocytes, also oscillate [70]. Serial bone marrow examination reveals a wave of cell production propagating successively from the colony forming units to the mature cells in each lineage [79, 39, 38, 67, 81, 1]. The stem cell origin of this disorder is strongly suggested by the fact that it can be transferred and cured by bone marrow transplantation [84, 92]. Both human and canine cyclic neutropenia are reviewed in [27, 29, 94, 95, 158, 70].

Human recombinant granulocyte colony stimulating factor (rhG-CSF) has been successfully used in severe chronic neutropenia to increase the nadir

ANC to normal levels and to prevent recurrent infections [68, 124, 15, 211]. Studies of the differential blood counts of five CN patients further showed an increase in the amplitude of the oscillations during G-CSF administration coupled with a decrease of the period of the oscillations from 21 days to 14 days in all the cell lineages [68]. In another patient, probably having acquired CN, G-CSF abolished the oscillations [68]. There have been some reports of oscillations in the ANC of congenital and idiopathic neutropenic patients during G-CSF administration [124, 15].

We report here on an extensive analysis of serial blood counts from 45 neutropenic patients with cyclical, congenital or idiopathic neutropenia who were treated with G-CSF during a phase III clinical trial [33]. Using periodogram analysis, we tested for the presence of statistically significant periodic oscillations in the serial blood counts of the patients before and during treatment with G-CSF, and determined these periods.

3.2 Data and Methods

Adult and pediatric patients with severe idiopathic neutropenia, cyclic neutropenia, or congenital neutropenia were evaluated, including some cases of congenital agranulocytosis (Kostmann's syndrome), Schwachman-Diamond syndrome, and myelokathexis from a randomized controlled clinical trial [33]. Patients with congenital agranulocytosis (Kostmann's syndrome) had severe neutropenia recognized soon after birth because of recurrent fevers and infections. Typically, the marrow showed some early neutrophil precursors,

but very few cells beyond the promyelocyte stage, often with accompanying increased eosinophils in the marrow. In most cases no other family members were affected. Patients were diagnosed as having Schwachman-Diamond syndrome if they had severe congenital neutropenia associated with findings of pancreatic insufficiency. Patients with myelokathexis had severe leukopenia, as well as neutropenia, with very pycnotic nuclei in the blood neutrophils. Patients with cyclic neutropenia had three to five days of neutrophils less than 0.5×10^9/L at regularly spaced, usually three week intervals over a six month period. Idiopathic neutropenia was the category for patients lacking symptoms or findings of neutropenia during the first years of life, but developing these changes later in life. Generally, the severity of neutropenia and the associated complications were less severe in patients with idiopathic neutropenia than in the other categories.

Eligible patients were then randomized to one of two treatment groups. One group began a four month observation period after which they received G-CSF therapy for five months. The other group received the same treatment, begun immediately. G-CSF was administered subcutaneously in doses sufficient to raise the blood neutrophil count to between 1.5×10^9 and 10×10^9 cells/L, with dose adjustments to maintain counts in this range. Serial blood cell counts were performed two to three times per week throughout the study period [33].

We used the Lomb periodogram to detect periodicity in the blood counts before and during treatment with G-CSF [104]. The Lomb periodogram is

equivalent to power spectrum analysis but is tailored for unevenly sampled data sets [104, 171]. In the case of discrete time series, the periodogram is calculated for a discrete number of frequencies. For each frequency, the value taken by the periodogram is inversely correlated with the distance between the data set and a sinusoidal wave of the same frequency, independently of the amplitude and phase. Scargle showed that the periodogram is indeed equivalent to least-square fitting of the data to a sum of sinusoids [171]. The statistical significance (p value) of any peak in the periodogram, i.e. the probability of finding such a value if the data was pure gaussian noise, is derived from the statistical distribution of the periodogram. We used the Lomb periodogram instead of the normal periodogram because of its simple statistical behavior in the case of unevenly sampled data [171].

Specifically, let x_j be the number of a particular type of cell as measured at times t_j, where $j = 1, \cdots, N$ and N is the number of data points. As usual, the mean and variance of the data values are given by

$$\bar{x} \equiv \frac{1}{N}\sum_{i=1}^{N} x_j \qquad \sigma^2 \equiv \frac{1}{N-1}\sum_{i=1}^{N}(x_i - \bar{x})^2. \tag{3.1}$$

Then the *Lomb normalized periodogram* $P(T)$ at a period T is defined by

$$P(T) \equiv \frac{1}{\sigma^2}\left\{\frac{\left[\sum_{j=1}^{N}(x_j - \bar{x})\cos\frac{2\pi(t_j-\tau)}{T}\right]^2}{\sum_{j=1}^{N}\cos^2\frac{2\pi(t_j-\tau)}{T}} + \frac{\left[\sum_{j=1}^{N}(x_j - \bar{x})\sin\frac{2\pi(t_j-\tau)}{T}\right]^2}{\sum_{j=1}^{N}\sin^2\frac{2\pi(t_j-\tau)}{T}}\right\}, \tag{3.2}$$

where the constant τ is defined implicitly by

$$\tan\left(\frac{4\pi\tau}{T}\right) = \frac{\sum_{j=1}^{N}\sin(4\pi t_j/T)}{\sum_{j=1}^{N}\cos(4\pi t_j/T)}. \tag{3.3}$$

The value of P(T) indicates the likelihood of a periodicity with period T in the data set. We implemented Equation 3.2 for a series of different periods T. A judgment must be further made as to whether or not there is a period for which the power P(T) is significantly high. The estimation of the significance level of P(T) is straightforward as long as some rules are followed for the choice of the range and the number of periods T that are scanned [171, 156]. We implemented an adaptation of the procedure proposed in [156] using Matlab. Copies of this program are available from the authors for the analysis of analogous data.

Once a significant periodicity T has been detected through periodogram analysis, the estimation of the phase (ϕ) and amplitude (A) of the sine wave that fits best the data can be calculated by a simple linear least-square fitting procedure [104, 171] of the data x_j, using

$$x = A \sin\left(\frac{2\pi t}{T} + \phi\right) \tag{3.4}$$

The estimated values of A and ϕ satisfy:

$$\frac{d(\sum_j (x(t_j) - x_j)^2)}{dA} = 0 \tag{3.5}$$

and

$$\frac{d(\sum_j (x(t_j) - x_j)^2)}{d\phi} = 0 \tag{3.6}$$

for $j = 1, ..., N$.

Note that there is an uncertainty in the estimation of the period since only a discrete set of periods can be tested.

3.3 Results

Of the 45 patients we analyzed, 38 had significant periodicity at the $p = 0.1$ level in at least one cell type, before or during treatment with G-CSF. Tables 1 and 2 summarize the results of all of our determinations of significant periodicities and their levels of significance (indicated only if $p \leq 0.10$) for the neutrophils, lymphocytes, monocytes, and platelets.

Figures 3.1 and 3.2 show the Lomb periodograms for the serial blood counts of five CN patients, three ConN patients and two IN patients, before and during treatment with G-CSF. Peaks in the periodogram can occur in one or more cell lines in all three groups of patients. In some patients, there is no peak in the periodogram of the ANC but a clear peak in the periodogram of another cell line (patients 50 and 102 before treatment and patient 58 during treatment with G-CSF). In other patients (patients 104, 43, 114, and 80 before treatment and patient 102 during G-CSF treatment), the presence of a peak at the same period in the periodograms of two or more different cell types strongly suggests significant periodicity even though the power may not be significant for a given cell type.

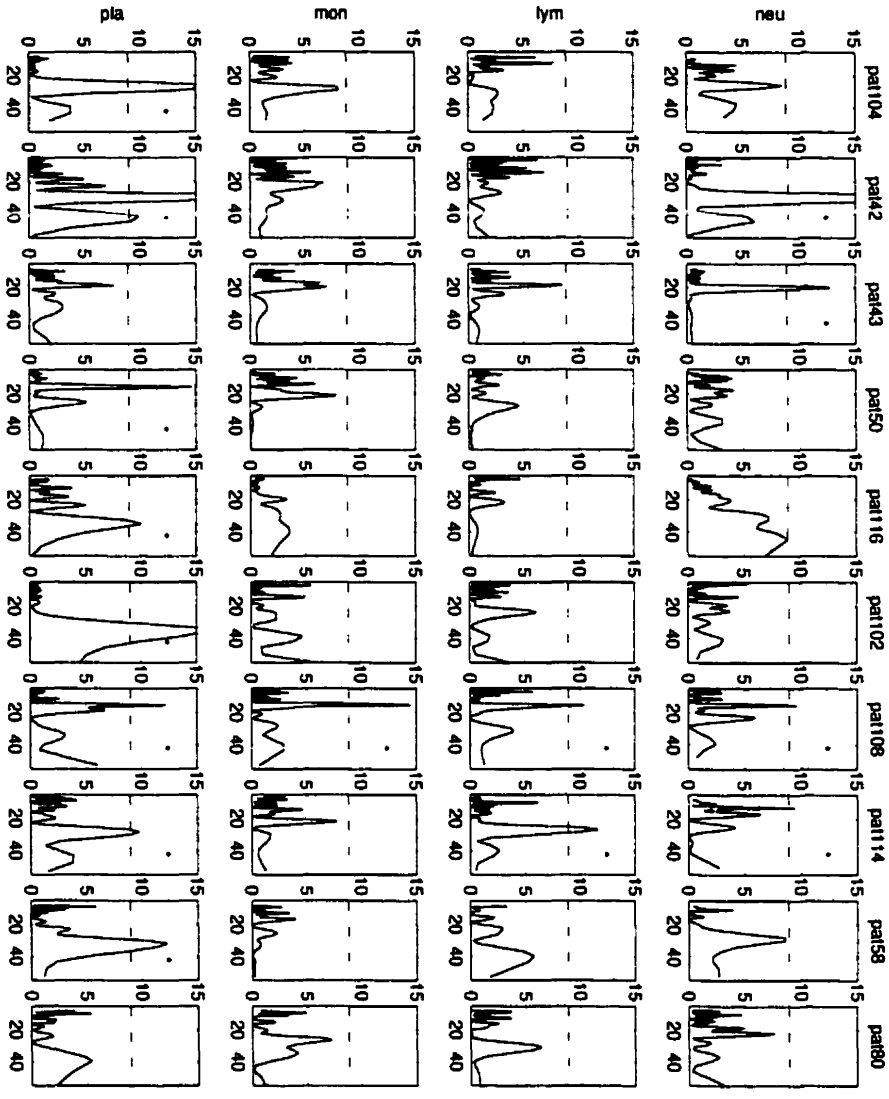

Figure 3.1: Lomb periodogram $P(T)$ [power P versus period T in days] of the blood cell counts of five CN, three ConN and two IN patients before treatment. The dotted lines in the Lomb periodogram give the $p = 0.10$ (lower dash-dot line) and $p = 0.05$ significance levels (upper dotted line); * indicates periodicity with significance $p \leq 0.10$. 'Neu': neutrophils, 'Lym': lymphocytes, 'Mon': monocytes, 'Pla': platelets.

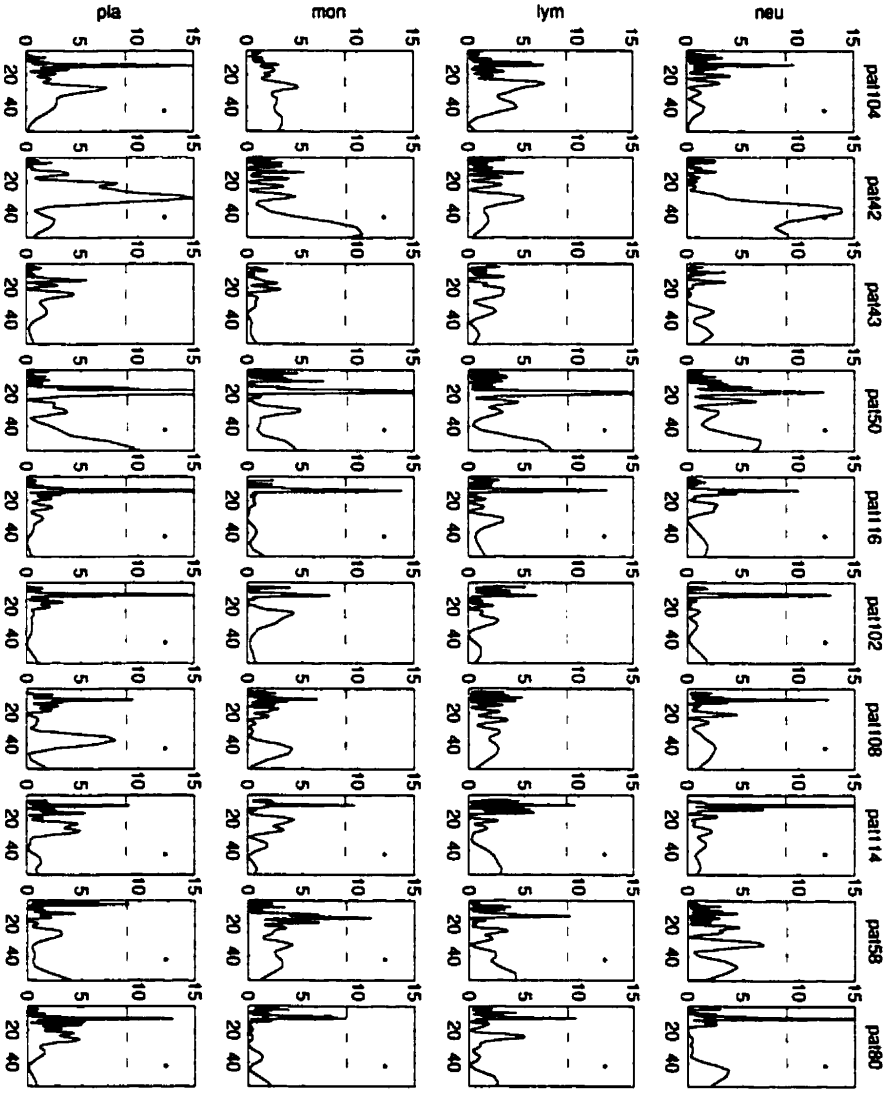

Figure 3.2: Lomb periodogram of the blood cell counts of the same five CN, three conN and two IN patients shown in Figure 1 but during G-CSF treatment. All symbols as in Figure 1.

3.3.1 Occurrence of periodicity in the ANC before treatment

We found significant ($p \leq 0.10$) periodicity in the ANC of nine patients, of which three were CN patients, two were ConN patients and four were IN patients. The ANC period was between 18 and 30 days in four of these (three CN and one IN). In the remaining five patients (two ConN and three IN) with significant periodicity, the periods found were 11, 13, 15 , 46 and 52 days respectively. Clearly, cyclicity is a property shared by all three patient groups and the criteria for establishing the diagnosis are insufficient to predict the presence or absence of statistically significant periodicity in the ANC.

3.3.2 Occurrence of periodicity in the other cell types before treatment

Of the nine patients that showed significant periodicity in the ANC, two patients (one CN, one ConN) also showed significant oscillations with the same period in one or more other cell type (platelets, monocytes and/or lymphocytes). The periods of the oscillations in these patients were 30 days (patient 42) and 15 days (patient 108) respectively. Even if the periodogram analysis clearly indicates periodicity in all the cell types as in patient 108 (see Figure 3.3), it may be hard to detect it by visual inspection of the serial blood counts because of the irregular sampling frequency and the high level of noise.

In two patients, oscillations occurred in different cell lineages with dif-

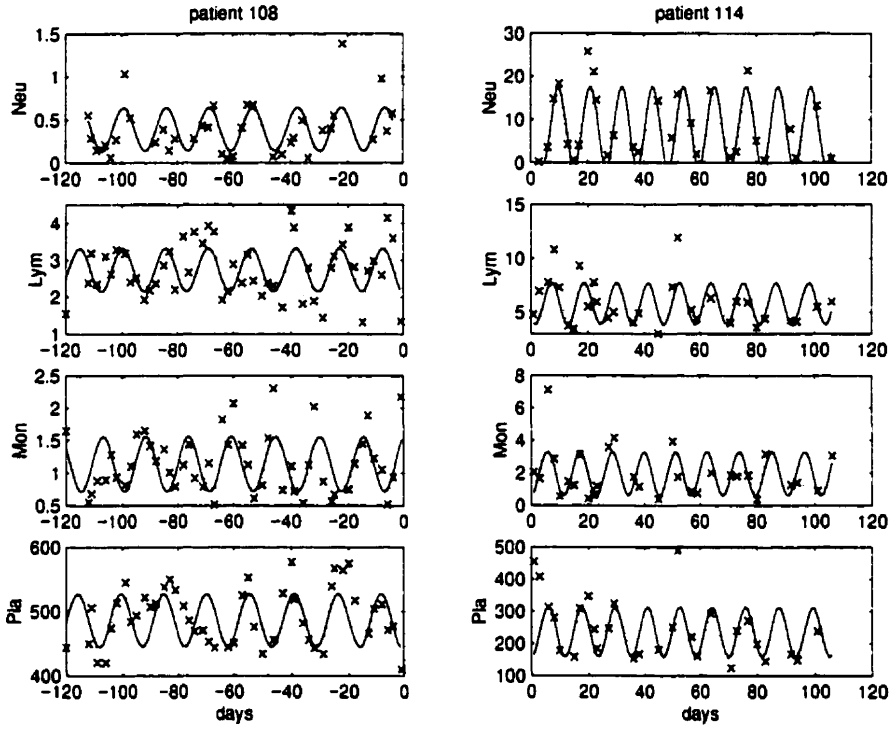

Figure 3.3: Sine wave fitting of the differential blood counts of congenital neutropenic patient 108 before treatment and congenital patient 114 during treatment with G-CSF.

ferent periods: In patient 54, the period was 18 days in the neutrophils and 12 days in the monocytes; and in patient 114, the period was 13 days in the neutrophils, 26 days in the lymphocytes and 28 days in the platelets. A similar difference in periods between cell types in the same patient has been previously reported in a CN patient [61], where the neutrophils oscillated with a period of 20 days and the platelets oscillated with a period of 35 days.

Significant oscillations in the lymphocytes, monocytes or platelets can also occur independently of oscillations in the ANC. Of the 35 patients with no periodicity in the ANC before treatment, 17 patients had significant periodicity in one or more other cell lineages, with periods ranging from 13 to 44 days (cf. Table 1).

Overall we detected significant periodic oscillations in one of the blood cell lines in 26 of the 46 neutropenic patients before treatment. Figure 3.4 shows the histogram of the periods (T) found in all the cell lines before treatment. Even though periodicities between 20 and 25 days are the most frequent, the occurrence of periods outside this range is also quite common. There are no discernible differences between the three diagnostic groups since $11 \leq T \leq 44$ for CN, $13 \leq T \leq 52$ for ConN, and $11 \leq T \leq 46$ for IN.

3.3.3 Effect of G-CSF on the periodicity in the ANC

Modification or abolition of ANC periodicity with G-CSF

Three of the nine patients showing periodicity in the ANC before treatment (two ConN, one IN) had a reduction in their periods during G-CSF

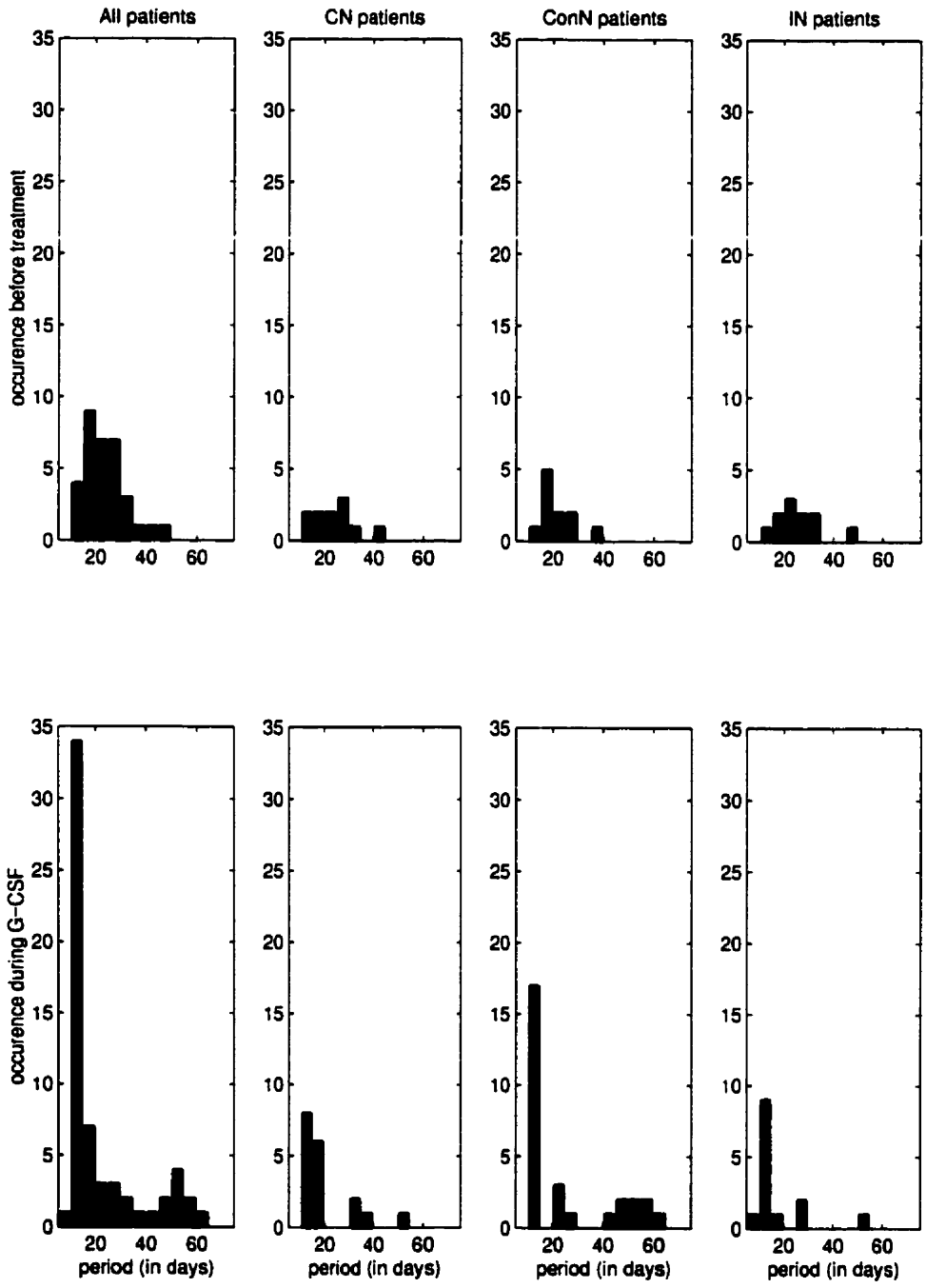

Figure 3.4: Histograms of the significant periods found in all the patients in the neutrophils, lymphocytes monocytes and platelets, before and during treatment with G-CSF.

treatment (cf. Table 2). Before treatment, these three had periods $13 \leq T \leq 46$ days, while during treatment $11 \leq T \leq 14$ days as reported previously [198, 68]. In two cases (patients 42 and 26) the period *increased* during G-CSF administration from 30 days to 39 days and from 11 to 25 days respectively. Furthermore, G-CSF therapy abolished significant oscillations in four patients (two CN and two IN). This effect was also described earlier [68].

Induction of ANC oscillations with G-CSF

In 16 patients (five CN, nine ConN, two IN), the data of Tables 1 and 2 show that G-CSF induced significant oscillations in the ANC where none existed before treatment. Eleven of these patients had ANC oscillations with periods $11 \leq T \leq 14$ days, which is the same range reported for the period of CN patients treated with G-CSF [68], while the other five had periods of 17, 19 (two patients), 52 and 64 days.

3.3.4 Effect of G-CSF on the oscillations in the other cells

During G-CSF treatment, 16 patients had significant oscillations both in the ANC and in another cell lineage, five patients had oscillations only in the ANC, and 10 patients had no oscillations in the ANC but significant oscillations in another cell lineages. The period in these latter 10 patients ranged from 7 days to 58 days. Of these patients, five had no significant periodicity in any cell type before G-CSF treatment. As with the neutrophils, G-CSF could either abolish, modify or induce significant oscillations in all

the blood cells. Periodicity during G-CSF treatment is thus not dependent on the existence of periodicity before treatment.

Figure 3.3 shows the cycling of all the cell lineages with a period of 11 days during G-CSF administration in patient 114. Before treatment with G-CSF, the neutrophils were oscillating with a period of 13 days and the lymphocytes and the platelets were oscillating with a period of 26 days. Administration of G-CSF induced significant periodic oscillations in the monocyte counts and decreased the period of the oscillations in all the other cell types.

The histograms of the significant periods found during G-CSF treatment shows an increase in the occurrence of significant oscillations and a concentration of the periods in the 10 to 15 day range for all the diagnostic groups (see Figure 3.4).

3.4 Discussion

The typical pattern of CN is easily recognizable because of large fluctuations in the ANC from very low to normal values. The period of the oscillations in the ANC of human CN patients is typically taken to be in the range of 19–21 days [27]. Other reviews have suggested a larger range of periods (18 to 30 days) [94, 158], and a case of neutropenia with a period of 100 days has been reported [12]. There has been one description of a case of human CN with a period of 14 days [43].

The results of the periodogram analysis reported here show that:

- The occurrence of significant ANC cycling in neutropenics not clas-

sified as cyclical neutropenic is much more prevalent than had been previously thought.

- Not all the patients classified as cyclic show significant ANC periodicity.
- Periodicity occurs before and during G-CSF treatment as frequently in the platelet counts as in the ANC, and less often in the other cell types (although the platelet, monocyte and lymphocytes counts are normal). In several patients significant periodicity occurred in platelets and/or monocytes and not in the neutrophils, especially before treatment with G-CSF. Thus, the occurrence of significant cycling in at least one (neutrophil, lymphocyte, monocyte, or platelet) cell line is much more common than is usually thought to be the case with neutropenic patients (10 of the 11 CN patients showed cycling in at least one of these lines, as did 17 out of 20 ConN patients, and 12 out of 14 IN patients);
- The range of periods encountered in these patients is much broader (in this study the range of significant periods involving any one of the cell lines shows that $11 \leq T \leq 52$ days) than is usually associated with classical cyclical neutropenia. Periods larger than 60 days may be due to long trend effects associated with the start of G-CSF administration.
- Significant periodicity was more frequent during G-CSF treatment than before treatment, and the periods found were not as uniformly spread between 10 days and 50 days: a majority of the oscillations found

during treatment ranged between 11 days and 14 days. Also, G-CSF increased the number of cell types showing periodic cycling per patient.

These observations suggest that "cyclical hematopoiesis", broadly defined as the presence of statistically significant cycling in at least one cell line, is probably highly prevalent in neutropenic patients (39 of the 45 patients of this study fell into this definition). However periodicity may be detectable only during G-CSF treatment. When irregular sampling occurs, periodicity can sometimes be very hard to detect without the use of periodogram analysis. This may explain why we found significant periodicity in neutropenic patients that had not been classified as cyclic.

Since significant periodicity occurs before and during G-CSF administration in these three types of neutropenic patients, we conclude that the occurrence of periodic oscillations is very strongly linked to the cause of the neutropenia itself. Hematopoiesis is regulated by a complicated network of cytokines which are produced in part by the hemopoietic cells. It is thus very likely that the alteration of one of the major hemopoietic compartments will affect the regulation and the dynamics of early hemopoietic cells and/or the other cell lineages.

Studies of the serial neutrophil counts in normal individuals showed no significant cycling [32, 119]. Periodic cycling of the blood counts has been observed in other untreated hematological disorders associated with bone marrow defects such as chronic myelogenous leukemia and aplastic anemia, with periods between 30 and 100 days [70]. The present finding that oscil-

lations with periods as large as 52 days also occur in CN and other types of neutropenia sheds new light on the relation between these apparently disparate disorders. Similar mechanisms controlling the dynamics of pluripotential stem cells may be responsible for the occurrence of periodic fluctuations of the blood counts in these different disorders. The finding that CN can be a premalignant manifestation of acute lymphoblastic leukemia [99] supports the hypothesis that there are common mechanisms involved in separate hematological disorders.

The effects of G-CSF on the dynamics of the various cell lines showed a variety of patterns ranging from modification (usually a decrease) of the period of oscillation in the ANC or other cell lines, an abolition of any significant periodicity, an initiation of a significant periodicity, or no effect on periodicity.

G-CSF is the only cytokine known to affect the period of the oscillations in CN. GM-CSF led to an increase in neutrophil count by a factor of between 1.5 and 3.5 – much less than achieved by G-CSF – and obliterated all signs of the cycling [211]. Although recombinant canine SCF (rc-SCF) did not cause neutrophilia in the grey collie, it did obliterate the oscillations of CN. Lithium therapy in grey collies [69, 67] and humans with CN [177, 192] has uniformly yielded an elimination of the severe neutropenic phases, and a diminution in the amplitude of the oscillations without any apparent change in the period of the oscillation.

It is likely that the modification in the period of the oscillations is a

consequence of an effect of G-CSF on the pluripotential early hematopoietic stem cell (HSC), since several blood lineages are affected simultaneously, while the increase in the mean white blood cell counts can be explained by the specific effect of G-CSF on the amplification of white blood cells precursors.

Diagnosis	#	Neutrophils	Lymphocytes	Monocytes	Platelets
CN	104	-	-	-	27 ± 1.8 (0.001)
CN	39	-	-	-	-
CN	42	30 ± 1.7 ($4.9e^{-8}$)	-	-	30 ± 1.7 ($3e^{-6}$)
CN	43	20.2 ± 0.9 (.009)	-	-	-
CN	50	-	-	-	16 ± 0.5 (.002)
CN	69	-	-	-	13 ± 0.3 (0.07)
CN	116	-	-	-	34.8 ± 2.7 (0.06)
CN	25	-	-	20.4 ± 0.7(0.08)	-
CN	18	-	44.4 ± 1.9 (0.07)	-	-
CN	54	17.8 ± 0.8 (0.04)		11.8 ± 0.3 (0.07)	-
ConN	10		-	-	
ConN	102	-	-	-	35.7 ± 2.7 (0.001)
ConN	105	-	-	-	22.4 ± 1.1 (.008)
ConN	106		-	-	-
ConN	108	15.4 ± 0.5 (0.01)	15.4 ± 0.5 (0.02)	15.4 ± 0.5 (0.003)	15.4 ± 0.5 (0.009)
ConN	11	-	-	-	-
ConN	114	13 ± 0.4 (0.07)	26.4 ± 1.6 (0.02)	-	28 ± 1.8 (0.06)
ConN	32	-	-	20 ± 0.9 (0.005)	52.4 ± 5.2 (0.08)
ConN	4	-	-	-	22.5 ± 1.1 (0.002)
ConN	60	-	-	-	-
ConN	63	-	-	-	-
ConN	66	-	-	-	-
ConN	74	-	-	-	-
ConN	95	-	-	-	26.4 ± 1.6 (0.04)
ConN	97	-	-	-	17 ± 0.6 (0.03)
ConN	115	-	-	-	-
ConN	111	-	-	-	27 ± 1.8 (0.07)
IN	103	-	-	24 ± 1.3 (0.08)	32.6 ± 2.5 (0.004)
IN	29	-	-	-	-
IN	58	-	-	-	31.7 ± 2.3 (0.02)
IN	7	46.2 ± 4.6 (0.05)	-	-	-
IN	77	19.8 ± 0.9 (0.03)	-	-	-
IN	78	-	-	-	20.7 ± 0.9 (0.05)
IN	80	-	-	-	-
IN	98	-	-	-	22.7 ± 1.1 (0.05)
IN	23	52 ± 5.2 (0.008)	-	-	-
IN	24	-	-	-	-
IN	26	11.2 ± 0.3 (0.05)	-	-	-

Table 1: Periodicities (in days) estimated through periodogram analysis (± the uncertainty inherent to periodogram analysis) with their significance level (in parenthesis), when the significance levels $p \leq 0.10$, before treatment with G-CSF (CN: Cyclic, ConN: Severe congenital, IN: Idiopathic, - : not periodic: / : not enough data).

Diagnosis	#	Neutrophils	Lymphocytes	Monocytes	Platelets
CN	104	13.7 ± 0.3 (0.06)	-	-	14.4 ± 0.4 (0.001)
CN	39	17.4 ± 0.5 (0.03)	-	-	17.9 ± 0.6 (0.04)
CN	42	38.9 ± 2.8 (0.003)	-	53.1 ± 5.3(0.03)	30.1 ± 1.7 (0.001)
CN	43	-	-	-	-
CN	50	18.7 ± 0.6 (0.008)	19.1 ± 0.6 ($2e^{-4}$)	18.5 ± 0.6 (2.8e-5)	18.5 ± 0.6 ($1.8e^{-15}$)
CN	69	11.8 ± 0.3 (0.05)	-	-	11.3 ± 0.2 (0.03)
CN	116	13.6 ± 0.4 (0.02)	13.3 ± 0.3 (0.008)	13.7 ± 0.3 (0.004)	13.7 ± 0.3 ($1.2e^{-05}$)
CN	25	-	-	-	33.8 ± 2.3 (0.06)
CN	18	-	-	-	-
CN	54	-	-	-	-
ConN	10	10.8 ± 0.2 (0.002)	-	-	-
ConN	102	12.7 ± 0.3 (0.007)	-	-	12.7 ± 0.3 (0.002)
ConN	105	52.4 ± 5.8 (0.07)	11.9 ± 0.3 (0.09)	-	12.5 ± 0.3 (0.02)
ConN	106	64 ± 8.0 (0.04)	-	-	-
ConN	108	11.5 ± 0.2 (0.006)	-	-	11.8 ± 0.2 (0.04)
ConN	11	52 ± 5.2 (0.02)	-	47.7 ± 4.3 (0.08)	-
ConN	114	11.4 ± 0.2 (0.0004)	11.1 ± 0.2 (0.03)	11.4 ±0.2 (0.05)	11.6 ± 0.2 (0.06)
ConN	32	11.3 ± 0.2 (0.02)	-	-	-
ConN	4	-	-	-	58 ± 5.8 (0.08)
ConN	60	12.4 ± 0.3 (0.04)	12.4 ± 0.3 (0.08)	-	28.6 ± 1.5 (0.02)
ConN	63	-	-	-	24.2 ± 1.2 (0.09)
ConN	66	12.9 ± 0.3 (0.04)		-	12.9 ± 0.3 (0.02)
ConN	74	-	-	-	58.4 ± 6.5 (0.05)
ConN	95	11.7 ± 0.2 (0.01)	-	-	-
ConN	97	-	-	56.4 ± 6.3 (0.01)	-
ConN	115	/	-	22.3 ± 0.9 (0.04)	22.3 ± 0.9 (0.007)
ConN	111	-	-	41.8 ± 3.5 (0.07)	-
IN	103	-	-	-	-
IN	29	-	-	-	50.9 ± 5.1 (0.04)
IN	58	-	15.6 ± 0.4 (0.07)	15.9 ± 0.5 (0.02)	7.4 ± 0.1 (0.05)
IN	7	12.4 ± 0.3 (0.0002)	-	-	12.4 ± 0.3 (0.001)
IN	77	-	-	/	-
IN	78	13 ± 0.3 (0.03)	-	-	13.2 ± 0.3 (0.003)
IN	80	12.7 ± 0.3 (0.0009)	12.4 ± 0.3 (0.04)	12.4 ± 0.3 (0.09)	12.7 ± 0.3 (0.003)
IN	98	/	-	-	-
IN	23	-	-	-	-
IN	24	-	-	25.6 ± 1.2 (0.07)	-
IN	26	25.2 ± 1.3 (0.07)	-	-	-

Table 2: Periodicities (in days) estimated through periodogram analysis during G-CSF treatment. All symbols as in Table 1.

Chapter 4

Haematopoietic Dynamics in Grey Collies

4.1 Introduction

Normally, the regulation of hematopoiesis maintains functional levels of white blood cells (WBC), red blood cells (RBC) and platelets in the blood. Though these cells play separate and independent functions, it has been shown that they all arise ultimately from a common primitive pluripotential stem cell (PPSC) population. Most disorders of hematopoiesis regulation lead to chronic failures of the production of either all the blood cell types or only one cell type. Models of these disorders are based on the existence of either lineage–specific regulators [e.g. erythropoietin (EPO), granulocyte colony stimulating factor (G-CSF), thrombopoietin (TPO)] or multilineage regulators such as stem cell factor (SCF).

Cyclical neutropenia (CN) is a peculiar disorder which does not correspond to either of these categories. It is characterized, in humans and in

grey collies (GC), by recurring episodes of neutropenia occurring at regular intervals. It is thus a dynamical disorder [52] rather than a constant defect. Moreover, other cell types (platelets, reticulocytes, monocytes and, more rarely, lymphocytes and eosinophils) often oscillate with the same period as the neutrophils [61]. The monocyte, eosinophil, platelet, and reticulocyte oscillations are generally from normal to high levels, in contrast to the neutrophils which oscillate from near normal to extremely low levels. Thus this disorder affects several cell lineages in different ways.

Transplantation studies suggest that the origin of the defect in CN is resident in one of the stem cell populations of the bone marrow [26, 83, 84, 197, 92, 153]. Studies of bone marrow cellularity throughout a complete cycle in humans with CN show that there is an orderly cell density wave that extends back into the CFU-G [79], the CFU-E [39, 38, 67, 81] as well as in the BFU-E and CFU-GM [1, 67]. Studies in the grey collie [6, 108] and in humans [66, 212] show that the responsiveness of granulocyte committed progenitor cells to G-CSF is greatly attenuated in CN compared to normal. In CN the levels of colony stimulating activity (CSA, related to G-CSF) fluctuate inversely with the circulating neutrophil levels [30, 60, 129]. It is unclear if this is related to the cause of CN, or is a consequence of the ANC oscillations.

The complex aspects of CN have led to conflicting hypotheses about its origin, and have generated a variety of mathematical modeling studies. One group of models hypothesizes a destabilization of the peripheral control of

granulopoiesis mediated by granulocyte colony stimulating factor (G-CSF) to be the origin of the periodic episodes of neutropenia [86, 89, 109, 134, 135, 132, 133, 160, 178, 182, 172, 173, 174, 175, 176, 202]. There is evidence that G-CSF mediates a negative feedback loop controlling granulopoiesis [70]. However, a recent modeling study indicates that this peripheral control is probably stable in CN and thus cannot account for the occurrence of oscillations [73].

A second group of models proposes that there is a destabilization of a pluripotential stem cell population since oscillations occur in all the blood cell lineages. Mackey [110, 112] proposed that there could be a loss of stability in the stem cell population independent of feedback from peripheral circulating cell types. The existence of cell to cell interactions mediated by cytokines which control the growth of hematopoietic stem cells (HSC) in vitro, such as interlukin-3 (Il-3) and stem cell factor (SCF), supports the hypothesis of an autoregulation of hemopoietic stem cells [141, 142, 143, 144, 55, 58, 59, 139, 138, 145, 162, 163, 164].

To better understand the relationship between the regulation of the different blood cell lineages in CN, we analyzed serial blood counts from 9 GC's. We used power spectral analysis to detect and quantify the periodic components of the serial counts. We show that the dynamics of the serial blood counts in GC can be understood by a model including an oscillatory output from the PPSC common to all the cell lineages and a peripheral feedback specific to the neutrophils.

4.2 Data collection and methods

The grey collie dogs were housed in individual cages and temperature-controlled quarters for these studies. Normal mongrel dogs served as the controls. Blood specimens were routinely drawn from the cephalic vein of unanesthetized dogs between 8:30 and 9:30 a.m. White blood cell and platelet counts were performed on EDTA anticoagulated blood specimens with a Coulter Counter (Coulter Electronics, Hialeah, Florida). One-hundred-cell differential counts were performed on air dried, Wright's stained smears. Purified rhG-CSF and rcG-CSF provided by AMGEN Inc. (Thousand Oaks,CA) were administered subcutaneously once daily.

We used the Lomb periodogram to detect periodicity in the blood counts [104], as has been described previously in [71]. The Lomb periodogram is equivalent to Fourier power spectral analysis but is tailored for unevenly sampled data sets [104, 171]. In the case of discrete time series, the periodogram is calculated for a discrete number of frequencies. The statistical significance (p value) of any peak in the periodogram can be calculated [171]. For each frequency, the value taken by the periodogram is inversely correlated with the sum of the squares of the residuals of the fit of the data set to a sinusoidal wave with frequency f. The presence of a significant peak at one of the frequencies f implies that the data set is periodic with a period $T = 1/f$.

We implemented an adaptation of the procedure proposed in [156] using

Matlab. Copies of this programme are available from the authors for the analysis of analogous data for noncommercial purposes.

Estimation of the phase and amplitude of the sine wave (or the sum of sine waves) that best fits the data was calculated by linear least-square fitting as in Chapter 3 [71].

Integrations of the model were done by the Runge-Kutta method using XPP with an integration step size of 0.05 days (freeware written by Prof. B. Ermentrout, see the web site at http://www3.pitt.edu/ phase/).

4.3 Analysis of serial blood counts in grey collies

4.3.1 Pattern of oscillations in different cell types

Figures 4.1 and 4.2 show the differential blood counts and the Lomb periodogram of the differential blood counts for nine GC's and one normal dog. Periodogram analysis shows periodicity of the absolute neutrophil count (ANC) and the platelet counts in all the GC's, and of the monocyte counts in eight of the nine GC's, with frequencies (f) between 0.065 ($T = 12.5$ days) and 0.080 ($T = 14.5$ days). We detected significant periodic oscillation of the lymphocyte and/or eosinophil counts in only two dogs (GC8 and 9). There was no significant periodicity in the serial blood count of the three normal dogs analyzed (data shown for one normal dog only).

The periodogram analysis shows the presence of harmonics in the ANC of GC 3 to 9, represented by peaks at twice the main frequency (f), and

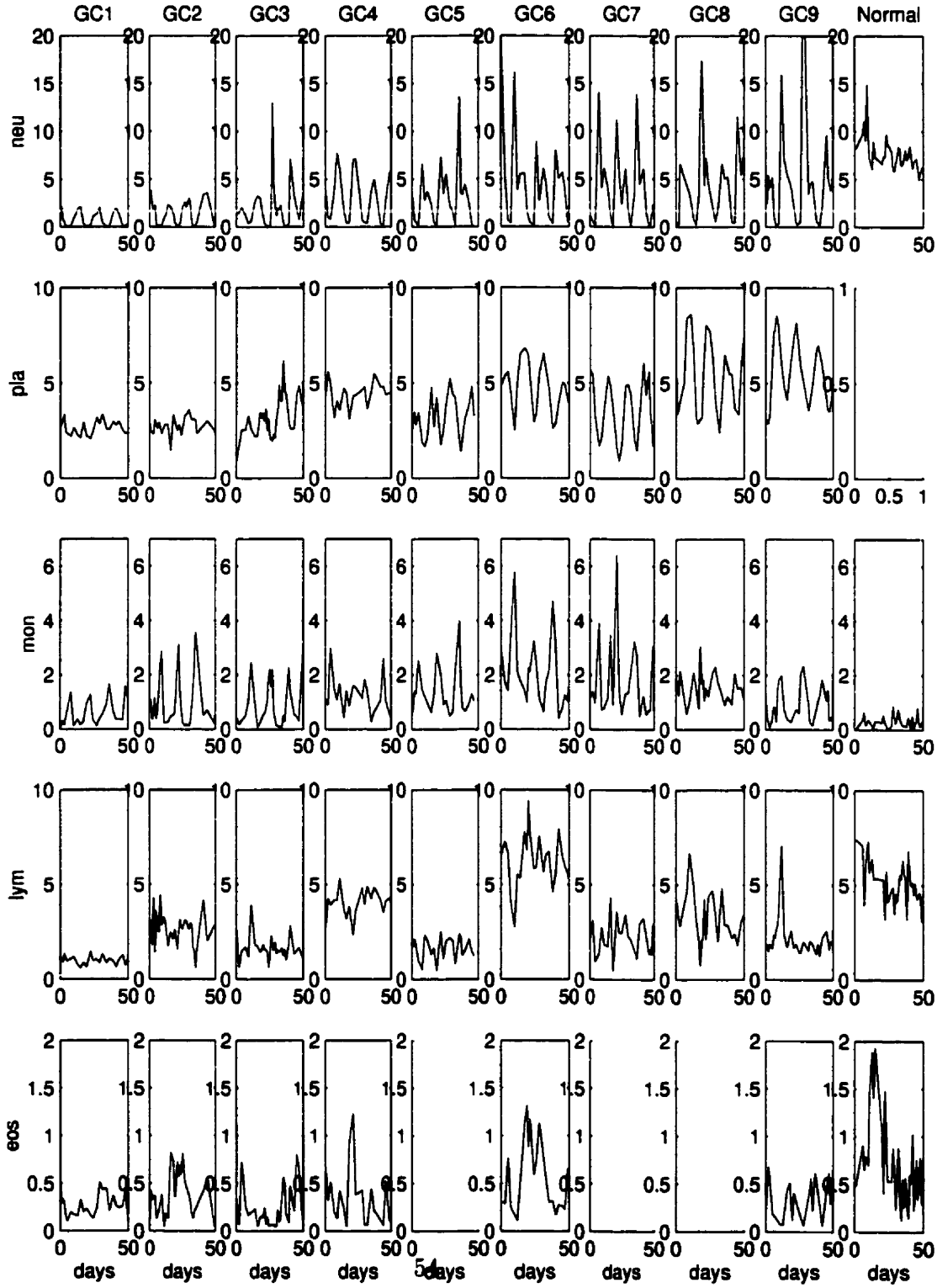

Figure 4.1: Differential blood counts in nine grey collies and one normal dog. Units: Cells $\times 10^{-5}$ per mm^3 for the platelets and Cells $\times 10^{-3}$ per mm^3 for the other cell types.'Neu': neutrophils, 'Pla': platelets, 'Mon': monocytes, 'Lym': lymphocytes, 'Eos': eosinophils.

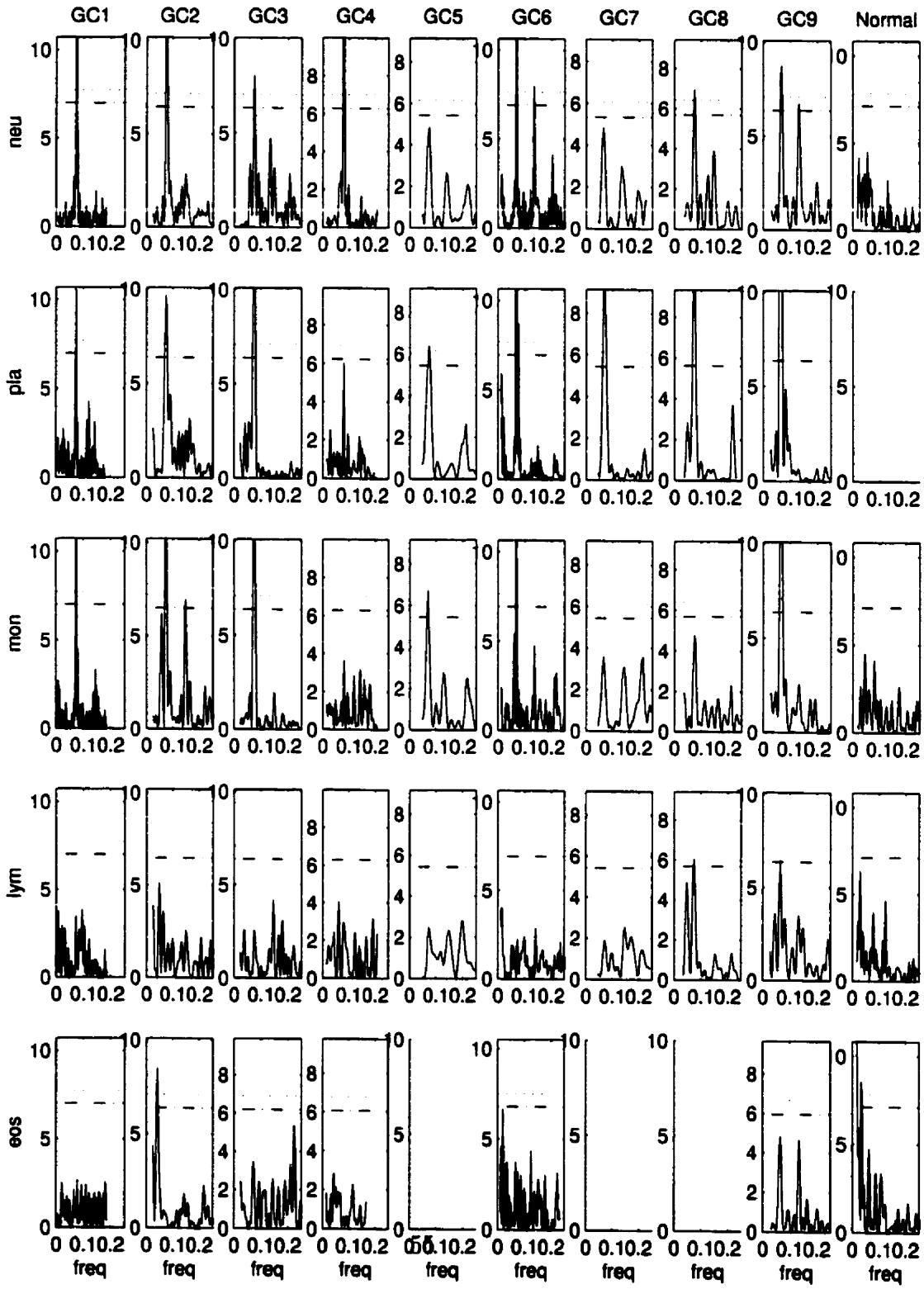

Figure 4.2: Lomb periodogram of the differential blood counts in nine grey collies.

sometimes at three times the main frequency (GC 3,5,6,7,9). The presence of these harmonic components implies that the pattern of the oscillations can be represented by a sum of sinusoidal waves with frequencies f, $2f$, $3f$ etc. Figure 4.3 shows the periodogram of GC 6 and the fitting of the serial ANC using the primary frequency and the second and third harmonics. We estimated the amplitude of the first (a_1), second (a_2), and third (a_3) harmonic components of the ANC by fitting the serial counts to the sum of three sine waves with frequencies f, $2f$, and $3f$ respectively.

Peaks at the harmonic frequencies occur in some GC in the other granulocytic lineages (e.g. monocytes and eosinophils) but never in the periodogram of the platelet counts. The platelet oscillations can thus be fit by a single sinusoid.

4.3.2 Variability of the dynamics in different grey collies

The presence of harmonics in the periodogram is not related to the period of the oscillations. We estimated the amplitude of the first (a_1) and second (a_2) harmonic components of the oscillations in the ANC in each dog by fitting the serial counts to the sum of two sine waves with frequencies f and $2f$. a_1 and a_2 both increase with the mean ANC, as does the ratio a_2/a_1 (Figure 4.4). There is thus a transformation of the oscillations in the ANC from a single sinusoid to a more complex oscillation that is correlated with the mean level of ANC and the amplitude of the oscillations (a_1). Such correlation

was not found for the other granulocytic cell lineages. The amplitude of the oscillations in the the neutrophils are also positively correlated with the amplitude of the oscillations in the platelets ($p \leq 0.10$). The change in the amplitude of the oscillations is thus not specific to the dynamics of the neutrophils, but is common to both cell lineages.

Variations in the amplitude of the oscillations not only occur in different GC's but can also occur in a single dog. Figure 4.5 shows the ANC and the platelet counts of GC 3 during a period where the dog was not receiving any treatment. A transition occurs at the third cycle in the ANC, from a low to high amplitude oscillation with the characteristic second bump. This transition is accompanied by an increase in the amplitude of the oscillations in the platelet counts. Individual GC's can thus express consecutively different dynamics of cyclic hematopoiesis during different temporal epochs.

4.3.3 Effect of G-CSF

As observed previously [65], G-CSF either abolished the cycling or decreased the period of the oscillations in all the cell lineages. In the latter case, the mean and amplitude of the oscillations was increased in the ANC and more mildly in the monocytes, whereas the mean and amplitude of the oscillations in the platelets was unchanged. In the dogs where the ANC showed harmonics (second bump), the latter disappeared during G-CSF administration (see Figure 4.6). In one dog which was administered G-CSF for an extended period, we observed an abolition of the oscillations in all the cell lineages

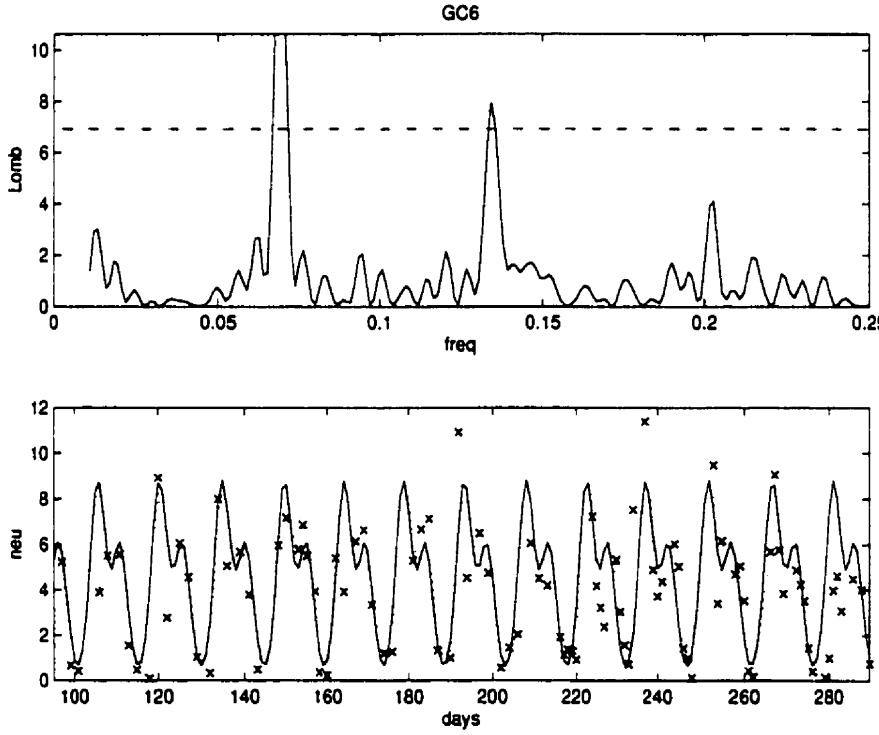

Figure 4.3: Lomb periodogram and fit of the ANC of GC 6. The function used to fit the data is $X(t) = 4.6 + 3\sin(2\pi ft - 0.6) + 1.8\sin(4\pi ft - 0.6) + 0.6\sin(6\pi ft - 3.2)$, with $f = 0.07$ (d^{-1}).

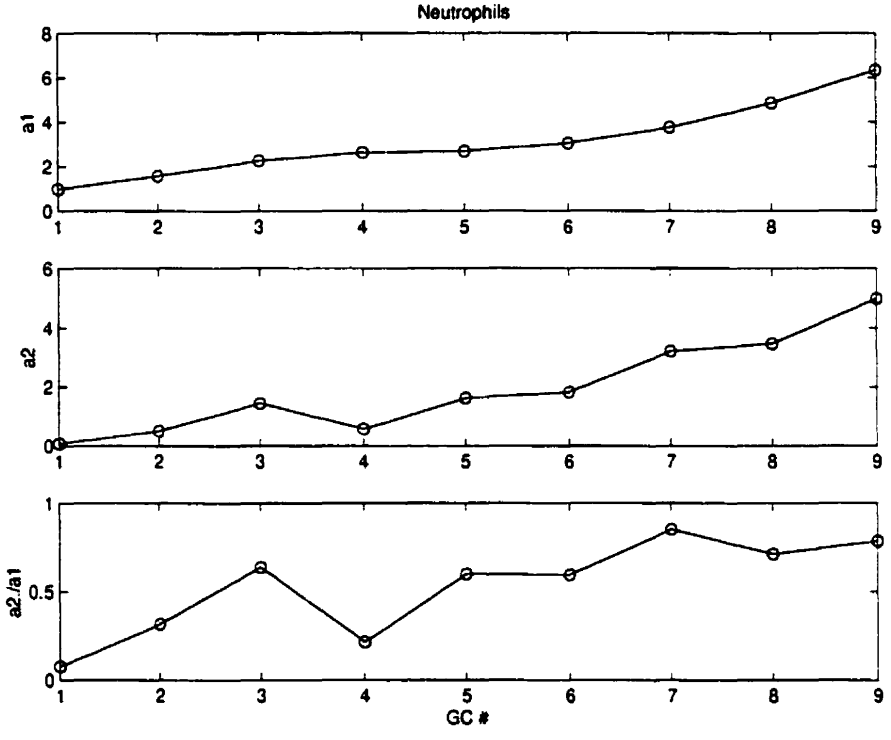

Figure 4.4: Variations of the amplitude of the first harmonic (a_1) and the second harmonic (a_2) in the absolute neutrophil counts (ANC) of the nine grey collies.

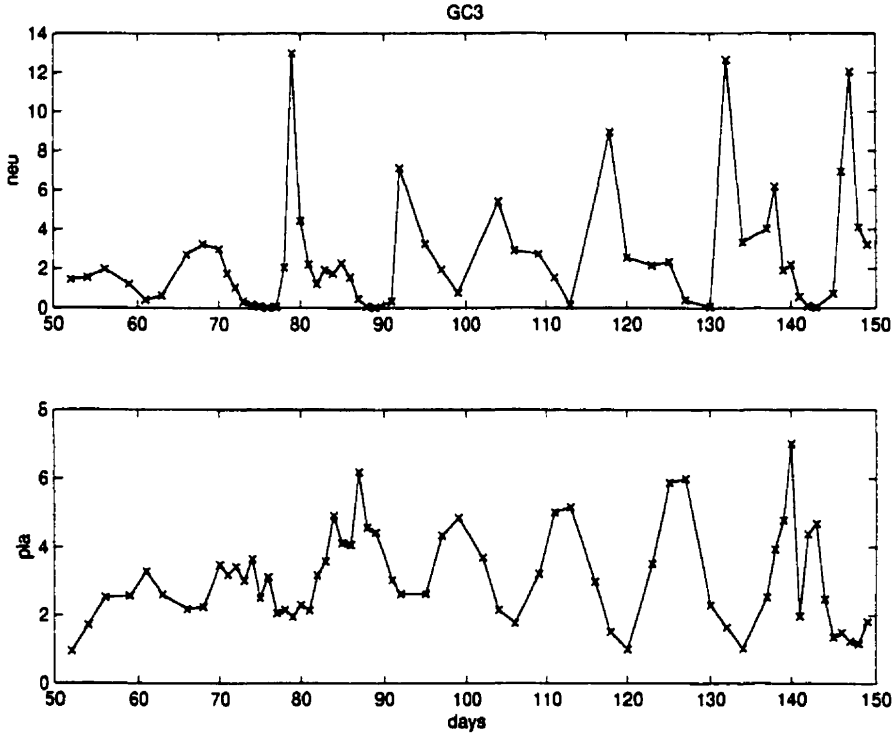

Figure 4.5: Neutrophil counts ($\times 10^{-3}$ per mm^3) and platelet counts ($\times 10^{-5}$ per mm^3) of GC 3. The data can be separated into two parts which have different dynamics.

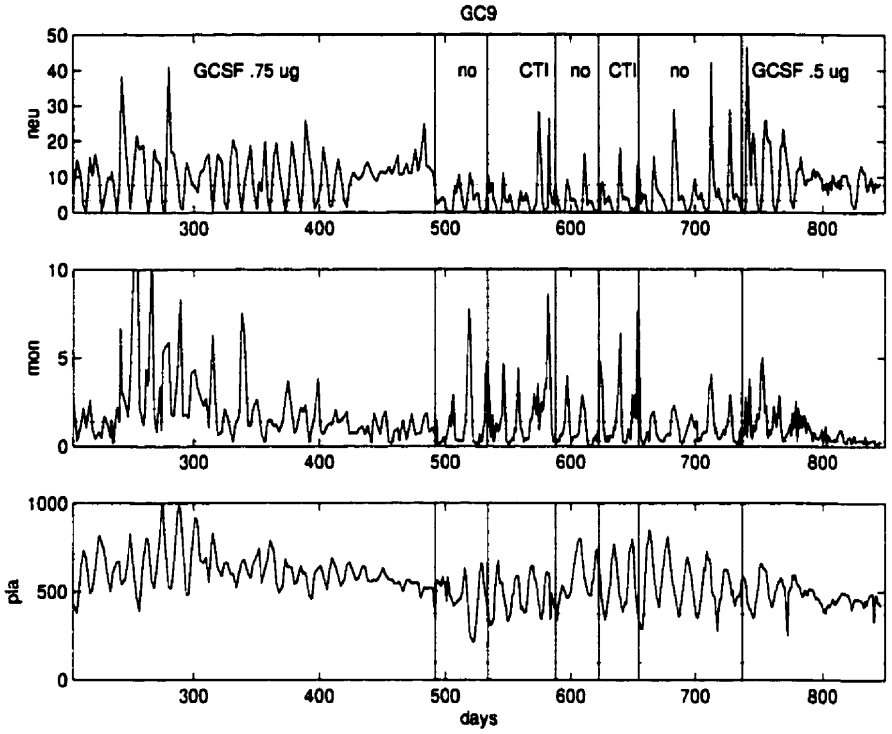

Figure 4.6: Serial blood counts of GC9, during treatment with G-CSF, CTI (CT-1501, provided by Cell Therapeutic Inc.) or no treatment. Dotted lines show normal ranges. Units: Cells $\times$ 10^{-5} per mm^3 for the platelets and Cells $\times$ 10^{-5} per mm^3 for the other cell types. 'Neu': neutrophils, 'Mon': monocytes, 'Pla': platelets.

more than 200 days after the treatment was started (see Figure 4.6). The dynamics of the serial blood counts during G-CSF administration is thus variable.

4.4 Model explanation of the neutrophil dynamics in grey collies

Given the occurrence of oscillations with the same period in all cell types in GC's, we assumed that the output from the PPSC is oscillating in GC's and that the oscillations propagate equally to all the blood cell lineages (see Figure 4.7). We assumed that the output from the PPSC was sinusoidal. The input from the PPSC to the granulocytic compartment is modulated by a peripheral feedback controlling the proliferation of the granulocyte precursors [73, 70]. This feedback is mediated by G-CSF which enhances the amplification of the granulocytic progenitors and which is inversely correlated with the levels of mature cells in the blood [96]. The amplification in the granulocytic compartment is thus a decreasing function of the number of mature neutrophils. Because of the time taken for granulocytic precursors to mature in the bone marrow, the effect of G-CSF on the peripheral density of neutrophils is delayed. The output from the granulocytic compartment into the blood is, therefore, a function of the sinusoidal input from the PPSC modified by a delayed negative feedback controlled by G-CSF. The delay represents the granulocytic maturation time, which ranges between 2.7 and 3.3 days [73].

There are also peripheral feedback loops controlling the amplification of the progenitors committed to the other cell lineages (for example mediated by EPO in the reticulocytes and TPO in the megakaryocytes). However,

since the cell density in these compartments oscillates within normal ranges, it is likely that the levels of regulators do not vary significantly (cf Figure 4.8).

The result of combining a sinusoidal input with the peripheral feedback control of granulopoiesis is shown in Figure 4.9. We used an exponential decreasing feedback with a delay of 3 days within the context of the model of [73].

We used the same feedback function for simulating the ANC in GC1 and GC6. For an input with a small amplitude the predicted oscillations in the neutrophil compartment are close to sinusoidal and fit the ANC of GC1. When the amplitude of the input is increased, the shape of the oscillations in the circulating neutrophils is transformed by the feedback function, giving the characteristic two peaks observed in the neutrophil counts of GC 3 to 9. The correlation between the model's prediction and the fit of the ANC using periodogram analysis is 0.95 for GC1 and 0.90 for GC6.

Absence of significant variations in the levels of TPO and other lineage-specific regulators implies that, unlike the ANC, the sinusoidal input to the megakaryocytes will not be distorted by the peripheral feedback. The amplitude of the oscillations in the peripheral blood counts will however increase with the amplitude of the input. This is consistent with the sinusoidal oscillations observed in the platelet counts and the positive correlation between the amplitude of the platelets oscillations and the amplitude of the neutrophils oscillations.

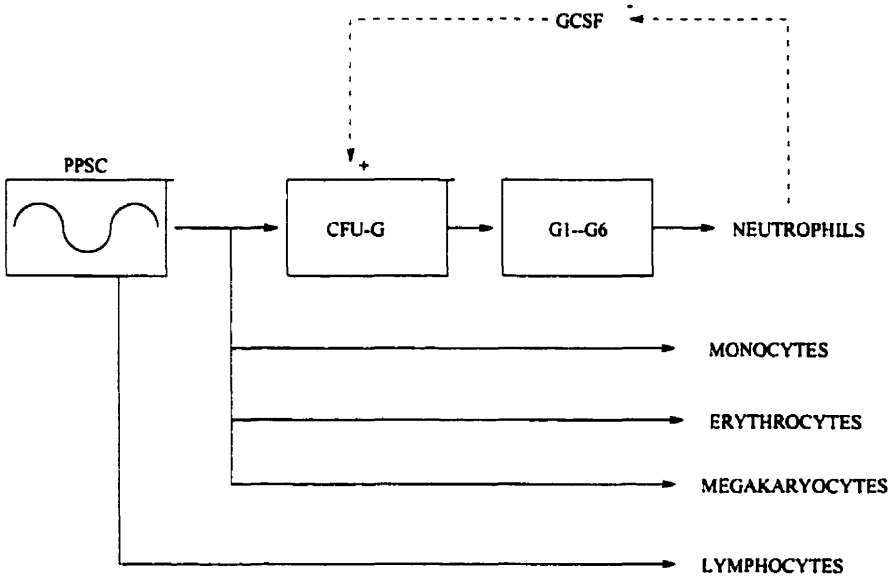

Figure 4.7: Scheme of granulopoiesis. The efflux from the pluripotential stem cells (PPSC) compartment differentiates towards granulopoiesis, erythropoiesis, megakaryopoiesis and lymphopoiesis. The granulopoietic lineage is represented by the colony forming unit committed to the granulocytes and the monocytes (CFU-G) and by the recognizable proliferating precursors: myeloblasts (G1), promyelocytes (G2), myelocytes (G3); and the post-mitotic precursors: metamyelocytes (G4), bands (G5) and segmented neutrophils (G6). Mature neutrophils are released in the granulocytic compartment in the peripheral blood. The proliferation of CFU-G is regulated by a feedback loop via granulocyte colony stimulating factor (G-CSF). The level of G-CSF is inversely correlated with the density of neutrophils in the blood.

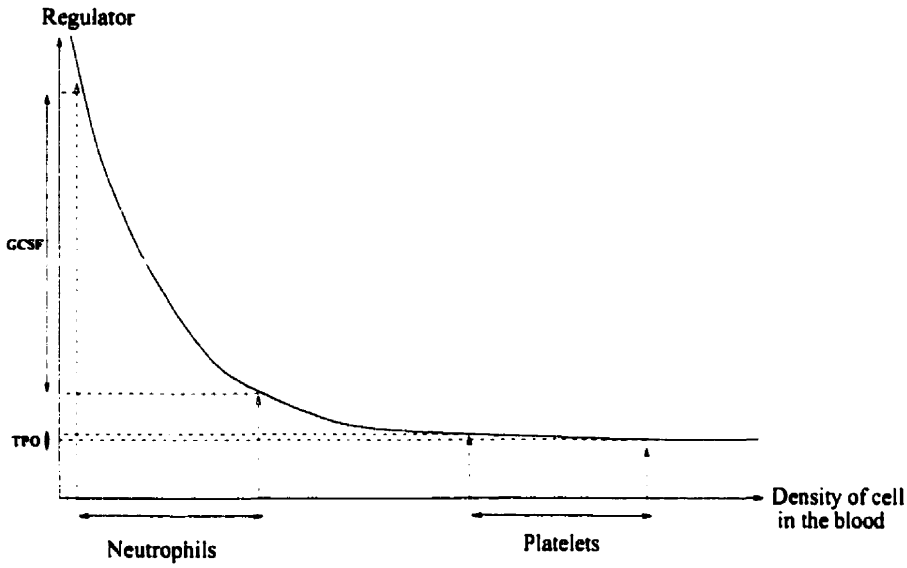

Figure 4.8: Illustration of the prediction of the model in Figure 4.7 regarding the variations of lineage-specific peripheral regulators in GC's, as a function of the density of cells, for the neutrophils and the platelets.

The increase in the mean and amplitude of the oscillations in the ANC during G-CSF administration can be explained by an increased amplification in the granulocyte progenitors compartment. The decrease in the period of the oscillations in all the cell lineages suggests that G-CSF also affects the dynamics of the PPSC. The fact that the mean and amplitude of the oscillations in the platelets are not modified by G-CSF suggests that both effects are independent and further supports the hypothesis that PPSC cycle independently of the peripheral control of granulocytes.

A much more detailed mathematical modeling study of the effects of increased oscillatory PPSC inputs on neutrophil dynamics is contained in [114].

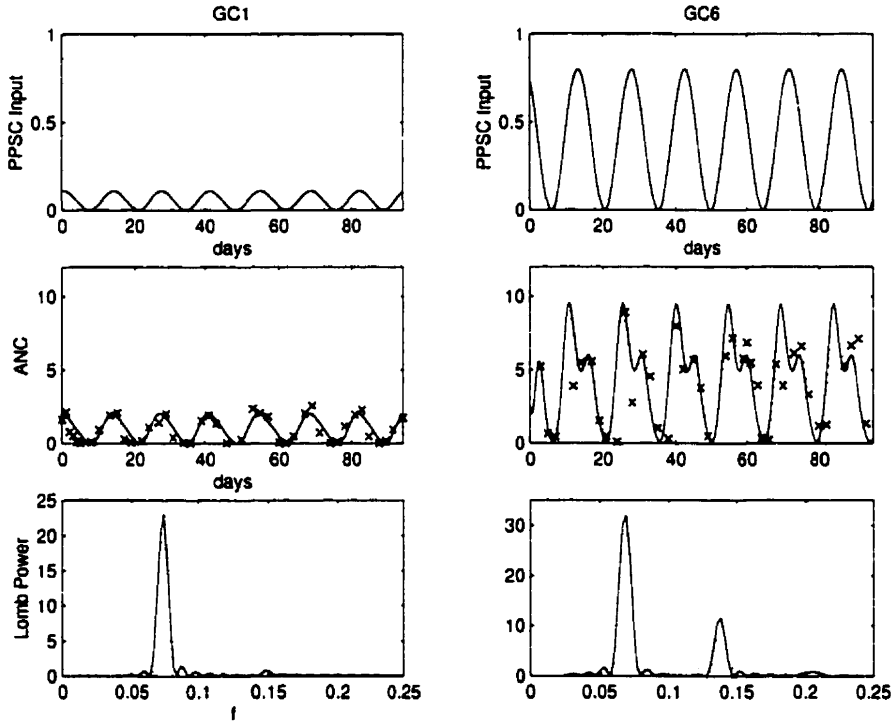

Figure 4.9: Simulations of the model of granulopoiesis. By varying the amplitude of the input from the PPSC, the predicted oscillations fit either the serial ANC of GC1 or GC6 (solid line: predictions of the model, dotted line, 'x': data points).

4.5 Discussion

The Lomb periodogram analysis allowed us to quantify variations in the dynamics of haematopoiesis in the GC, in particular in the neutrophils and the platelets. The amplitudes of the oscillations in these two cell lineages vary concomitantly. The power spectrum and the shape of the oscillations in the ANC vary together with the amplitude of the oscillations. As the amplitude of the oscillations increases, the height of the second harmonic increases, giving rise to a distorted oscillation with two peaks per cycle.

We showed that the particular dynamics of the ANC can be reproduced by a combination of a delayed peripheral feedback, representing the peripheral control of granulopoiesis through G-CSF, together with a sinusoidal input representing an oscillatory input from the PPSC to the granulocytic lineage. The distortion of the sinusoidal input by the peripheral feedback increases with the amplitude of the input. Thus the model predictions and the data are consistent.

The absence of distortion of the oscillations in the platelet counts can be explained by the absence of effective feedback in this cell lineage, that is the absence of significant variations in the levels of thrombopoietic regulators. This is consistent with the fact that the platelet counts oscillate within normal ranges. On the other hand, the ANC oscillate from normal to very low values, which induces dramatic changes in the levels of G-CSF. The other granulocytic cells and the monocytes may be also affected by the variations

in G-CSF, although more moderately.

Previous models of CN in the GC's did not take into account the differences in the dynamics of the different cell lineages and their variations. The present modeling study suggests that the peripheral feedback controlling granulocytosis is responsible for the distortion of the oscillations observed in the ANC. We could reproduce the dynamics of the different cell lineages by assuming a sinusoidal oscillation propagating from the PPSC into the different cell lineages independent of the peripheral regulation of the committed granulocytic progenitors. All the cell counts are oscillating within normal ranges except the neutrophil counts which are lower than normal. This is consistent with the defect observed in the growth of granulocytic progenitors in vitro, in GC's and in humans with congenital CN. The investigation of the precise mechanism that generates oscillations in the PPSC requires data on the kinetics of stem cell populations in the bone marrow.

Chapter 5

General Discussion

As reviewed in [70], models based on the hypothesis of a destabilization of the peripheral control of granulocyte production do not give a satisfying explanation of the features of CN. The occurrence in both human patients with CN and GC dogs of oscillations with the same period in several blood cell lineages suggests that the oscillations arise in the PPSC and propagate equally to all the cell lineages. Mackey [110] proposed that an increase in the death rate of PPSC is responsible for the onset of oscillations around a low average which would account for the low neutrophil counts. This hypothesis is however inconsistent with the fact that, except for the neutrophils, all the cell counts oscillate around or above normal values. We propose alternatively that the oscillations arise in the PPSC compartment but around a normal or slightly elevated average and that the neutropenia is due to a specific abnormality in the production of neutrophils. Moreover, we propose that the destabilization of the PPSC is a consequence of the neutropenia, as suggested by the data analysis of the neutropenic patients [71].

Bone marrow studies and stem cell transplantation studies in the GC's are consistent with the hypothesis of an oscillation in the PPSC compartment. Assuming both an oscillatory input from the PPSC and a peripheral feedback loop specific to the granulocytic cell lineage, we could also explain the dynamical features of CN in GC's [72].

In both humans and dogs, the data analysis suggests that G-CSF probably plays an important role in the destabilization of the PPSC population. In humans with neutropenia, administration of G-CSF may induce oscillations in all the cell lineages and in both CN patients and GC's, G-CSF administration modifies the period of the oscillations in all the cell lineages. Hemopoietic stem cells at early stages of development are dormant unless they are triggered to enter cell cycling. Il-3, Il-4 and GM-CSF regulate the proliferation of multipotential progenitors only after they are triggered to exit the dormant state. Triggering of cycling appears to require interactions of several cytokines such as Il-6, G-CSF, Il-11, Il-12, LIF and Steel Factor [147, 97]. In the model of the autoregulation of HSC presented in [110], G-CSF may thus affect the rate at which cells enter proliferation.

The amount of G-CSF free to bind to HSC depends on the amount of G-CSF administered or produced and also on the number of cells expressing G-CSF receptors at their surface [96]. The expression of the G-CSF receptor increases with the differentiation of the myeloid cells [181]. In normal individuals, G-CSF is thus bound mainly to granulocytes and granulocytic precursors in the blood and the bone marrow. Administration of G-CSF in

normals induces an increase in the granulocytic pool so that the amount of unbound G-CSF will not be increased. This clearance effect can explain that administration of G-CSF does not affect other cell lineages than the granulocytic and lymphoid lineages in normals. Conversely, knock-out of the G-CSF gene in mice obliterates the production of granulocytic cells but it does not seem to affect the HSC [101]. This can be explained by the overlapping effects of several other cytokines such as Il-11, Il-12, LIF and Steel Factor on the regulation of HSC which are thus not dependent on the presence of a single regulator. However, in neutropenic patients which have greatly reduced numbers of granulocytic cells, the amount of free G-CSF in the serum will be increased if the defect is not due to a decrease in the production of G-CSF. This excess of free G-CSF may be responsible for the destabilization of early HSC in CN. This is consistent with the observation that in CN patients and GC's where G-CSF serum levels were monitored, the latter were found to be increased.

One of the questions that has never been pointed out, to our knowledge, is the problem of the synchronization of the several populations of HSC. It is not clear yet whether hematopoiesis is maintained by one or a few clones, or a large number of clones simultaneously. If there are several populations of HSC sustaining hematopoiesis and if these populations are controlled by independent autoregulatory loops, the destabilization of these will induce oscillations with random phase lags in each hemopoietic clone. Thus the number of cells produced at a certain time in the whole body will be the

sum of several oscillatory functions with different phase lags. If the number of independent HSC populations is high, this will sum up to a constant production rate and no oscillations will be seen in the blood counts. Thus, in order for the blood cell densities to oscillate, one has to hypothesize that hematopoiesis is monoclonal or that the different populations of HSC are synchronized.

When the HSC are destabilized, the granulocytic cells and their precursors oscillate. The clearance effect implies that the amount of free G-CSF in the serum of the whole body will also oscillate, out of phase with the granulocytes. This is consistent with the observation that levels of colony stimulating activity (CSA-related to G-CSF) fluctuate inversely with the circulating neutrophil levels in the grey collie [30] and in humans [60, 129]. The triggering of cycling in the different HSC populations will thus be synchronized.

The hypothesis of an effect of G-CSF on the PPSC compartment may thus be necessary to explain the occurrence of peripheral oscillations.

Further advances in the biology of cytokines and their effect on the proliferation and differentiation of stem cells would allow us to build a realistic model of hematopoiesis, taking into account both the local regulation of HSC and the effect of serum G-CSF on the different hematopoietic compartments [147, 97]. One aspect of the regulation of hematopoiesis that is revealed in particular disorders such as CN, and that should be stressed, is the dynamical nature of the regulatory effects of cytokines. Because of the interdependence

of all the hematopoietic compartments, the effect of a regulator on a specific compartment may vary dramatically depending on the global state of the hematopoietic system. This has significant importance for the development of more efficient treatments for the various hematological disorders.

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