COMMUNAUTE FRANCAISE DE BELGIQUE
ACADEMIE UNIVERSITAIRE WALLONIE-EUROPE
FACULTE UNIVERSITAIRE DES SCIENCES AGRONOMIQUES DE GEMBLOUX

Measurement *in vivo* of cell turnover in patients with chronic lymphocytic leukaemia

Julien DEFOICHE

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Defoiche Julien (2009). Mesure *in vivo* de la dynamique cellulaire chez des patients atteints de la leucémie lymphoïde chronique (thèse de doctorat). Gembloux Faculté Universitaire des Sciences Agronomiques, **99 pages, 4 tableaux, 24 figures**.

Résumé : La leucémie lymphoïde chronique (LLC) est une maladie caractérisée par une accumulation et une infiltration clonale de cellules lymphocytaires (lymphocytes B) dans le sang, la moelle, les ganglions lymphatiques et la rate. Pendant longtemps on a pensé que la LLC était caractérisée par un taux de prolifération réduit, alors qu'au contraire une étude récente réalisée *in vivo* montre que ces cellules prolifèrent. De manière à répondre à ces divergences et de déterminer si les cellules LLC prolifèrent plus rapidement que les cellules B de patients sains, nous avons comparé et mesuré les paramètres dynamiques (taux de prolifération et de disparition) chez des patients LLC et sains en utilisant la technique du glucose deutéré. Par ailleurs, nous avons également estimé l'activité métabolique de ces cellules en mesurant le taux de renouvellement de l'ARN. Sur base de ces observations nous avons montré que les cellules B de patients atteints de la leucémie lymphoïde prolifèrent significativement moins que celles des personnes saines. De plus l'activité métabolique reflétée par le taux de renouvellement de l'ARN est significativement réduite chez les patients LLC.

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Summary: Chronic lymphocytic leukaemia (CLL) is a disease characterized by abnormal accumulation of B cells in the blood, bone marrow, lymph nodes and spleen. Several decades ago, it was concluded that CLL lymphocytes might be unable to proliferate *in vivo* but a recent study performed *in vivo* in patients with CLL has shown in contrast that these cells proliferate. However, an important and still unanswered question is whether CLL cells proliferate faster or slower compared to their normal counterparts. In this context, the turnover of CLL cell population was compared to the kinetics parameters of normal B lymphocytes after labelling with deuterium glucose. We have also compared the metabolic activity of CLL cells with B lymphocytes from healthy subjects using a new method for measuring RNA turnover *in vivo*. Based on these observations, we found that leukaemic cells proliferate less frequently than healthy patient and that metabolic activity *via* measurement of RNA turnover rate is significantly reduced in CLL patients.

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Abbreviations

ADPR-C	ADP-ribose cyclique
AMP	Adenosine monophosphate
Apaf-1	Apoptotic protease activating factor1
ATA	Aldonitrile triacetate derivative
ATM	Ataxia-Telangiectasia
Bcl-2	B-cell lymphoma 2
BCR	B-cell receptor
BH	Bcl-2 homology domain
BLNK	B-cell linker protein
BrdU	5'-Bromo-2'-deoxyuridine
Cdk	Cycline dependent kinase
CLL	Chronic lymphocytic leukaemia
DAG	Diacylglycerol
DED	Death Effects Domain
DD	Death Domain
dN	Pyrimidine nucleoside salvage pathway
dNDP	Deoxyribonucleotide diphosphate
dNTP	Deoxyribuncleotide triphosphate
ERK	Extracellular signal-regulated protein kinases
FADD	Fas Associated Death Domain
FCR	Fludarabine Cyclophosphamide Rituximab
FcγRIIB	Fc receptor for immunoglobulin
FCS	Fetal Calf Serum
GC/MS	Gas Chromatography Mass Spectrometry
GMP	Guanosine monophosphate
Hb	Haemoglobin
HPLC	High performance liquid chromatography
IAP	Inhibitor of apoptosis protein
ICAM	Intracellular adhesion molecules
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IgD	Immunoglobulin D
IGVH	Immunoglobulin variable region heavy chain
IMP	Inosine 5'- monophosphate
ITAM	Immunoreceptor tyrosine-based activation motifs (ITAM)
ITIM	Immunoreceptor tyrosine-based inhibitory motifs
IP3	Inositol-1,4,5-triphosphate
JNK	Jun N-terminal kinases
MAPK	Mitogen-activated protein kinase
MACS	Magnetic activated cell sorting
mRNA	messenger RNA
NDK	Nucleoside diphosphate kinases

NDP	Ribonucleotide diphosphate
NF-κB	Nuclear factor-kappa B
NLC	Nurse-like cells
NTP	Ribonucleotide triphosphate
PCI	Positif chemical ionization
PHA	Phytohemagglutinin
Pi3K	Phosphatidylinositol 3-Kinase
PIP2	Phosphatidylinositol-4, 5-biphosphate
PKC	Protein Kinase C
PLCγ	Phospholipase Cγ
PNAs	Purines nucleosides analogues
Pre-B cell	Precursor B cell
Pro-B cell	Progenitor B cell
PRPP	Phosphoribose-pyrophosphate
PTK	Protein tyrosine kinase
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
ROS	Reactive oxygen species
RR	Ribonucleotide reductase
SDF-1	Stromal derived factor
SIM	Selective ion monitoring
SHIP	src homology inositol phosphatase
TCR	T-cell receptor
TH	T helper cells
TNF	Tumor Necrosis Factor
TRAIL	Tumor Necrosis Factor Related Apoptosis Inducing Ligand
TRMA	Thiamine-responsive megaloblastic anemia
tRNA	Transfer RNA
VCAM-1	Vascular cell adhesion molecule-1
VLA4	Very Late Antigen-4
VPA	Valproic Acid
ZAP-70	Zeta-associated protein of 70 kda

Table of contents:

I. Introduction

1. Introduction

Chronic lymphocytic leukaemia (CLL) is a disease characterized by an accumulation of abnormal B cells due to imbalance between lymphocyte proliferation and death.

This thesis describes a series of investigations of cell turnover and metabolic aspects of chronic lymphocytic leukaemia. Prognostic markers and mechanisms involved in the accumulation of chronic lymphocytic leukaemia are described in the Introduction. The relevance of information about the cellular kinetic behaviour and lifespan of lymphocytes to our understanding of the mechanisms that regulate CLL is developed and discussed. The thesis goes on to demonstrate how a quantitative understanding of such immune based processes requires the development of experimental techniques able to estimate proliferation and death rates *in vivo*.

The second chapter of the thesis describes how the deuterium glucose method was used to quantify the turnover of lymphocytes in CLL. Next, it describes the development of an approach to measure RNA synthesis rates using a deuterium-labelled glucose approach. Finally, we describe a series of investigations of RNA turnover in CLL cells using this new approach.

2. Chronic lymphocytic leukaemia

2.1 Introduction

B lymphocytes mature in the bone marrow and the development of B cells begins when lymphoid precursor cells differentiate into the earliest distinctive B-lineage cell called progenitor B cell (pro-B cell) (Roitt I.M., 2002). At the earliest stage of the development, the binding of pro-B-cells with stromal cells in the bone marrow is mediated by several cell adhesion molecules including VLA-4 on pro-B cell and its ligand (VCAM-1) on the stromal cells. Following this interaction, the pro-B-cell divides and differentiates into a pre-B cell. During these intermediate stages, rearrangements occur at the level of immunoglobulin variable gene segments to create an immunoglobulin that serves as the B-cell receptor for the antigen. During antigen-independent maturation phase, immature B cells derived by differentiation of pre-B cells expressing IgM on their surface are generated in the bone marrow (Figure I.1). After this selection stage, the cells enter in the bloodstream and in lymph nodes and differentiate into mature naïve B cells expressing both IgD and IgM on their surface (Goldsby R.A., 2008) (Figure I.1). These naive B cells die unless they encounter antigens or activated T_H cells. In presence of T_H cells, activated B cells divide rapidly in germinal centres of lymph nodes and undergo somatic hyper-mutation in the V_HDJ_H and V_LJ_L gene segments producing a new antibody repertoire (Rajewsky, 1996) (Figure I.2). However marginal zone B cells respond to antigens without T cell participation (T-cell independent antigenic stimulation) and this pathway may or not induce V-gene mutations (Toellner *et al*, 2002) (Figure I.2). Finally, both pathways lead to the development of memory B cells or plasma cells.

The development of normal B cells into CLL cells can be explained by an initial mutation which occurs in a single B lymphocyte and this cell responds to self-antigens or other antigens. CLL is divided into two subgroups based on the presence or absence of somatic mutations in the immunoglobulin variable region heavy chain (*IGVH*) genes. It has been reported that CLL cells have a different distribution of *IGVH* genes (V_H1, V_H3 and V_H4 family genes) compared to normal peripheral blood $CD5^+$ B lymphocytes (Chiorazzi & Ferrarini, 2003), V_H1 and V_H3 being over and under expressed, respectively. Other mutations can occur causing the transformation of normal cells into CLL cells. Among these molecular changes, chromosomal abnormalities, such as deletions at 13q, 11q, 17p and duplication of chromosome 12 are involved in the development of the disease (Chiorazzi *et al*, 2005).

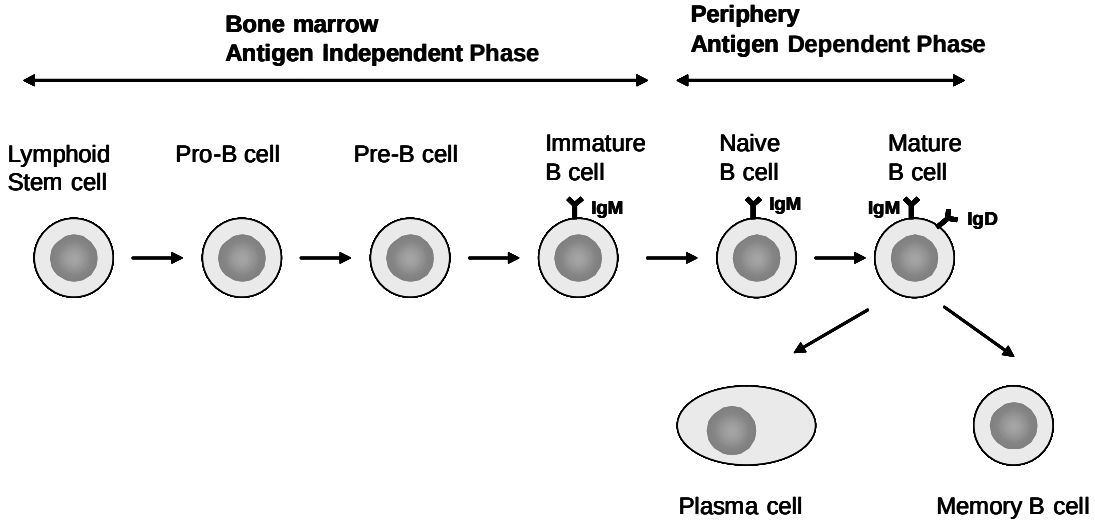

Figure I.1: Representation of stages of B-cell maturation (according to (Goldsby R.A., 2008)).

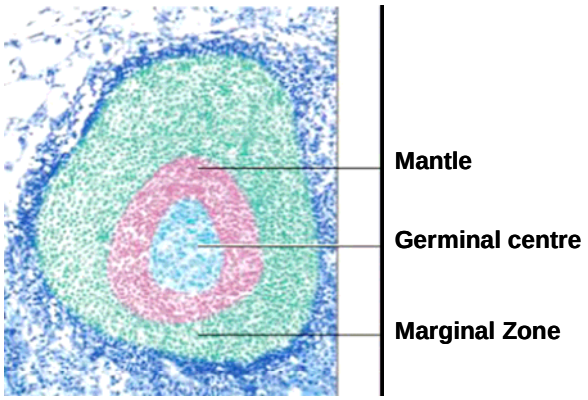

Figure I.2: Representation of lymph node follicle (according to (Chiorazzi *et al*, 2005)).

2.2 Definition

Chronic lymphocytic leukaemia (CLL) is a disease characterized by a monoclonal accumulation of small mature $CD5^+$ B lymphocytes in the blood, bone marrow, lymph node and spleen (Figure I.3), (Chiorazzi & Ferrarini, 2003;Oscier *et al*, 2004). CLL is the most common form of leukaemia in Western countries with an incidence ranged from less than 1 to 5.5 per 100.000 persons worldwide (Redaelli *et al*, 2004). CLL, which affects mainly individuals (90%) older than 50 years, is diagnosed when the lymphocyte count is greater than 10.000/μl (Bain *et al*, 2002) (Steine-Martin E.A, 1998).

In the last twenty years, CLL has also been observed in younger patients accounting for approximately 10-15% of cases, possibly due to an increasing number of routine diagnoses (De Rossi *et al*, 1989;Montserrat *et al*, 1991). Disease progression is correlated with a series of parameters including the mutation status of the immunoglobulin variable heavy chain (*IGVH*) genes, microRNA signature and expression of CD23, CD38 or ZAP-70 (Zeta-associated protein of 70 Kda) (Calin *et al*, 2005;Hamblin, 2005;Pekarsky *et al*, 2005;Stilgenbauer & Dohner, 2005). CLL has a highly heterogeneous clinical course with time to progression varying from months to many years (Hamblin, 2005). However a small proportion of cases can transform to a more aggressive form (Bennett *et al*, 1989) including (i) Richter syndrome (3 to 10% of cases), a poor prognosis lymphoma resistant to the most aggressive chemotherapy (Figure I.4), (Nakamura & Abe, 2003), or (ii) prolymphocytic leukaemia characterized by hepatomegaly and splenomegaly during which the white blood cell count can reach 100.000 cells per mm^3 of blood.

Chronic lymphocytic leukaemia is still an incurable disease, current treatments include a series of alkylating agents (Chlorambucil or Cyclophosphamide), purine nucleoside analogues (PNAs), which have primary effect on DNA synthesis (Fludarabine, Cladribine), and

monoclonal antibody anti CD20 (Rituximab). Currently, the most promising treatment for recurrent and refractory CLL includes fludarabine in combination with cyclophosphamide and rituximab (FCR) (Wierda *et al*, 2006;Keating *et al*, 2005).

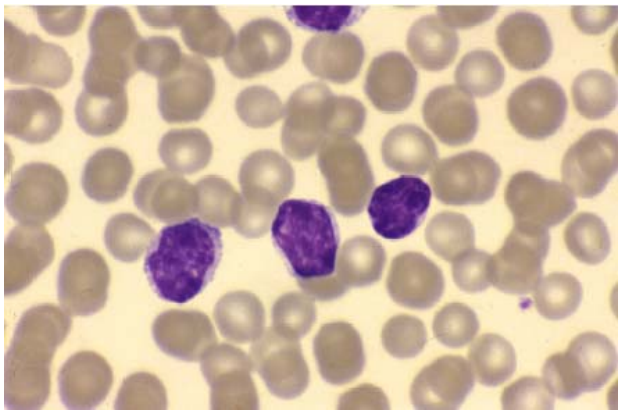

Figure I.3: Small/medium-sized lymphocytes from CLL stained with May-Grumwald-Giemsa (according to (Matutes *et al*, 2007)).

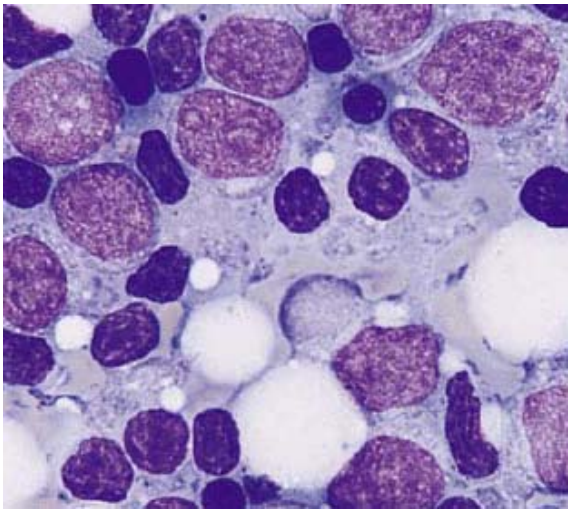

Figure I.4: Bone marrow of patients with Richter's syndrome, showing numerous large lymphoma cells and with small lymphocytes of chronic lymphocytic leukaemia (according to (Bain *et al*, 2002)).

2.3 Clinical Classification

Two clinical staging systems have been proposed to predict the survival of patients with CLL. (Binet *et al*, 1977;Rai *et al*, 1975). The Rai classification system designates five distinct stages (stages 0, I, II, III, or IV) whereas the classification according to Binet is defined in three stages including; the total lymphoid mass, the presence or absence of anaemia and thrombocytopenia correlated with median survival.

2.3.1: Rai clinical staging

Stage		Clinical features	Median Survival (years)
Original	Modified		
0	Low	Absolute lymphocytosis [(a)] of > 10,000/µl in blood and ≥ 30% lymphocytes in bone	>10
I	Intermediate	Lymphocytosis and lymphadenopathy [(b)]	6-8
II		Lymphocytosis and hepatomegaly [(c)]	
III	High	Lymphocytosis and anaemia (Hb < 11g/dl)	< 2
IV		Lymphocytosis and thrombocytopenia [(d)] (Platelets < 100 x 10^9/l)	

2.3.2: Binet clinical staging

Stage	Clinical features	Median Survival (years)
Original		
A	Lymphocytosis of with lymphadenopathy in < 2 LN regions	>10
B	Lymphadenopathy at > 3 sites in the absence of anaemia or thrombocytopenia	5
C	Anemia (Hb <10 g/dl) and/or thrombocytopenia (platelets < 100 x 106 /dl) with or without lymphadenopathy	2

(a) Lymphocytosis is defined as an increased of number of lymphocytes in the peripheral blood.
(b) Lymphadenopathy is an enlargement of the lymph nodes.
(c) Hepatomegaly is an enlargement of the liver.
(d) Thrombocytopenia is a reduced platelet count.

2.4 Immunophenotyping

CLL cells are mature B lymphocytes that express CD5, CD19 and CD23 as well as low levels of surface immunoglobulin (sIg); IgM, IgD and CD79b (Chiorazzi *et al*, 2005). In addition, all CLL cells express CD27 that characterizes the memory phenotype of B cells. Epitope specificity of transformed B cells includes self-antigens including anti-DNA, IgG, rheumatoid factor and red blood cells. CLL is a heterogeneous disease varying from months to many years which can be divided into two subgroups based on the presence or absence of somatic mutations in the immunoglobulin variable region heavy chain (*IGVH*) genes (Damle *et al*, 1999;Hamblin *et al*, 1999;Hamblin, 2002).

(i) CLL cells in patients with unmutated *IGVH* genes are characterized by high expression of ZAP-70 (≥20%) (Crespo *et al*, 2003), CD38 (≥30%) (Damle *et al*, 1999;Ghia *et al*, 2003) and telomerase (Chiorazzi & Ferrarini, 2003). These cells frequently harbour chromosomal abnormalities such as trisomy 12, deletions 11q22-23 and 17p13. In addition, CLL cells characterized by a lack of somatic mutations in the immunoglobulin variable regions are more sensitive to ligation of BCR complex (Lanham *et al*, 2003). These patients are characterized by an aggressive clinical course with a median survival of 8 years (Stilgenbauer *et al*, 2002).

(ii) Patients mutated for *IGVH* genes in contrast to unmutated subjects have a lower number of cells expressing CD38 (< 30%) (Damle *et al*, 1999;Ghia *et al*, 2003) and ZAP-70 (< 20%) (Crespo *et al*, 2003). These patients are more likely to have deletion on chromosome 13 occurring in approximately half of all CLL cases and usually do not require treatment for many years with a median survival of 25 years (Rosenwald *et al*, 2001).

2.5 The B-Cell Receptor (BCR)

The B-cell receptor (BCR) complex is composed of a surface membrane immunoglobulin most often IgM and/or IgD, associated with Ig-α (CD79a) / Ig-β (CD79b) heterodimer (Efremov *et al*, 2007). The cytoplasmic tails of CD79a/CD79b contain an immunoreceptor tyrosine-based activation motifs (ITAM) that acts as a docking site for kinases and adapter proteins involved in BCR signalling (Kipps, 2007). Once signal transduction is initiated through the BCR, B lymphocytes progress into the cell cycle or die. Signals delivered through the B-cell receptor are important for the outcome of the B cell. IgM signalling can induce either apoptosis or cell survival while signals delivered through IgDs extend cell survival (Zupo *et al*, 2002;Zupo *et al*, 2000;Bernal *et al*, 2001). Moreover, unmutated CLL cells are more sensitive to the ligation of BCR complex due to ZAP-70 that enhances BCR signalling of CLL cells (Chen *et al*, 2002; Chen *et al*, 2005).

Following the ligation of IgM by antigens, the BCR signalling pathway is activated. The first step starts when ITAMs of CD79a and CD79b are phosphorylated by src tyrosine kinase such as Lyn (Law *et al*, 1994) (Figure I.5). This leads to recruitment of tyrosine kinase such as Syk and ZAP-70 that phosphorylate the B-cell linker protein (BLNK) regulating the development and the outcome of B-cell function (Fu *et al*, 1998;Kabak *et al*, 2002). BLNK phosphorylation induces the activation of key signalling intermediates as phosphatidylinositol 3-kinase (PI3K) and phospholipase-Cγ (PLCγ) (Kurosaki & Tsukada, 2000;Kurosaki *et al*, 2000). Once activated, PI3K produces the phosphatidylinositol-3,4,5-triphosphate (PIP3), which recruits the kinase Akt. The PI3K/Akt pathway plays an important role in cell survival as Akt induces phosphorylation and inactivation of proteins involved in apoptosis such as Bad and caspase 9 (del Peso *et al*, 1997;Cardone *et al*, 1998).

In addition, activation of PLCγ induces catalytic activity for hydrolysis of phosphatidylinositol-4,5-biphosphate (PIP2) into diacylglycerol (DAG) and inositol-1,4,5-triphosphate (IP3). These two messengers mediate Ca^{2+} mobilization and activation of PKC. The signalling pathway continues with the activation of transcription factors including NF-kB and mitogen-activated protein kinases (MPAKs) like p38, ERK and JNK (Petlickovski *et al*, 2005;Efremov *et al*, 2007) (Figure I.5).

Conversely, the activation of BCR signalling pathway can be perturbed by the binding of cytoplasmic domains (ITIMs) of FcγRIIB to phosphatases such as SHIP (src homology-2-containing inositol phosphatase) that in turn can dephosphorylate activated signalling molecules and ITAMs of Ig (Kipps, 2007).

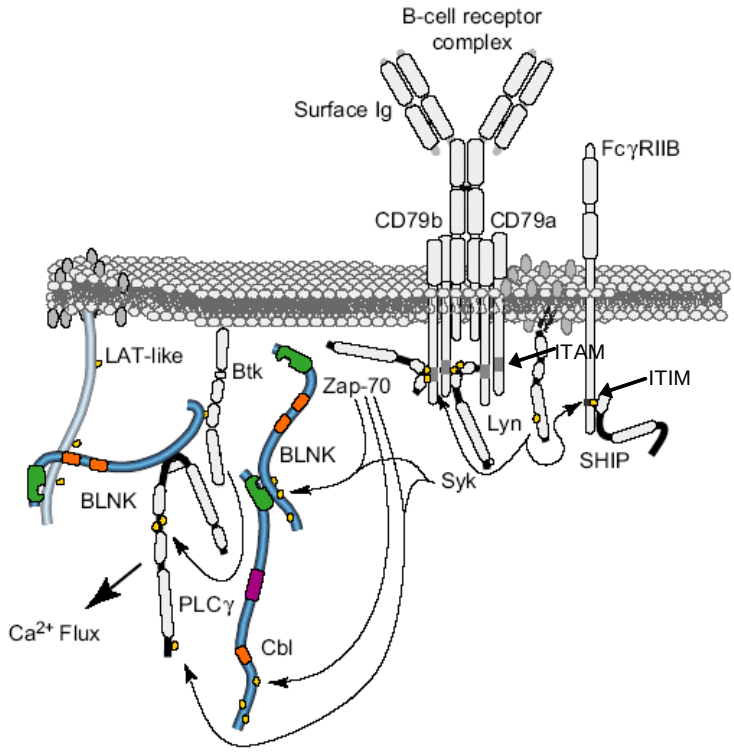

Figure I.5: B-cell receptor complex (BCR) in chronic lymphocytic leukaemia cell expressing ZAP-70 (according to (Kipps, 2007)).

2.6 Zeta-associated protein 70 (ZAP-70)

ZAP-70 (zeta-associated protein 70) stands for the 70-kDa cytoplasmic protein tyrosine kinase (PTK) that is characterized by 2 tandem src homology 2 (SH2) domains and a C-terminal catalytic domain. ZAP-70 is expressed in natural killer cells, T cells but also at various stages of B-cell maturation in normal pre-B-cells and pro-B cells (Kipps, 2007; Crespo *et al*, 2006).

In T cells, the ligation of the T-cell receptor (TCR) with its antigen induces the activation of a protein tyrosine kinase (Src-family) causing the phosphorylation of ITAMs. ZAP-70 is recruited and becomes activated causing the activation of downstream signalling pathways such as phospholipase C and Ras/MAPK pathway (Kane *et al*, 2000). In normal B cells, it is another related tyrosine kinase, Syk that is involved in B-cell receptor (BCR) signalling. ZAP-70 can complement BCR signalling in Syk-deficient B cells suggesting that Syk and ZAP-70 exert similar functions (Kong *et al*, 1995;Toyabe *et al*, 2001) (Figure I.5).

In CLL, cells expressing unmutated *IGVH* and ZAP-70 are more likely to respond to ligation of the BCR complex than those that do not express ZAP-70 (Dighiero & Hamblin, 2008). In fact, ZAP-70 enhances IgM signalling of CLL cells via Syk by phosphorylation of downstream signalling molecules such as BLNK (Chen *et al*, 2002). Moreover, ZAP-70 is detected in most CLL patients but at different levels of expression. In fact, CLL patients unmutated for *IGVH* are characterized by a level of expression of ZAP-70 greater than 20% whereas patients with mutated *IGVH* genes have less than 20% of ZAP-70 positive B cells. These unmutated *IGVH* B cells generally express levels of ZAP-70 proteins comparable to those expressed by normal blood T lymphocytes (Chen *et al*, 2002).

2.7 CD 38

CD38 is a transmembrane glycoprotein, NAD^+ glycohydrolase/ADP-ribosyl cyclase involved in transmembrane signalling and cell adhesion. The extracellular domain of CD38 contains an enzymatic site involved in the synthesis of cyclic adenosine diphosphate ribose (c-ADPR) from nicotinamide dinucleotide and hydrolyses c-ADPR to ADP ribose (Damle *et al*, 1999;Deaglio *et al*, 2005;Deaglio *et al*, 2006;Deaglio *et al*, 2008). Variations in expression of CD38 are associated with type II diabetes (Ikehata *et al*, 1998), HIV infection (Steel *et al*, 2008), prostatic cancer (Kramer *et al*, 1995), bone metabolism (Sun *et al*, 1999) and chronic lymphocytic leukaemia (Deaglio *et al*, 2005).

Expression of CD38 in CLL favours growth and survival of B cells via the activation of CD100 (a semaphorin family member) with its ligand and IL-2 signals. This signalling pathway is initiated when chemokines (SDF-1) induce the binding of CD38 to its ligand CD31, which is expressed by stromal and nurse-like cells. Following the ligation, the survival receptor CD100, which is expressed on CLL cells, is up-regulated and in turn binds to plexin-B1, a high affinity ligand which is found on stromal and nurse-like cells (Deaglio *et al*, 2005;Deaglio *et al*, 2006). The interaction between CD100 and its ligand extends the life span of CLL cells.

CD38 expression is an indicator of relatively poor prognosis (Hamblin *et al*, 2002) and its expression depends of the mutated status of *IGVH* genes. Low expression of CD38 expression occurs more frequently in the mutated B-CLL cases while higher expression occurs more frequently in the unmutated CLL cells. The expression of CD38 ($\geq$ 30%) is correlated to disease aggressiveness and shorter survival time (Ibrahim *et al*, 2001;Del Poeta *et al*, 2001). It has been also demonstrated that CD38 expression is lower on cells derived from blood than those from the bone marrow (Ghia *et al*, 2003).

2.8 Telomerase

Telomeres are indicators of cell division history; they are hexameric repeats (5'-TTAGGG-3') located at the end of chromosomes that shorten with each round of cell division. Telomere length is restored by telomerase, an enzyme whose reverse transcription activity is capable of adding TTAGGG sequences at the 3'ends of telomeres. The telomere lengths of B-CLL cells are shorter than those of age matched normal donors indicating that these leukaemic cells have prolonged proliferative history (Damle *et al*, 2004). Moreover telomere lengths of unmutated B CLL cases are much shorter than those of mutated cases. Studies suggest heterogeneity in telomerase expression in bone marrow samples of B-CLL patients (Bechter *et al*, 1998;Chiorazzi & Ferrarini, 2003); this might be related either to a greater number for cell divisions or reduced activity of telomerase. In fact, telomerase activity is significantly higher in unmutated B-CLL cells than mutated CLL cells suggesting that unmutated B-CLL cells have undergone many cell divisions even more than those of the mutated group (Damle *et al*, 2004;Chiorazzi & Ferrarini, 2003).

2.9 Cytogenetic abnormalities

Chromosomal abnormalities in CLL are associated with disease progression and survival. Patients with unmutated B-CLL cells have cytogenetic changes (e.g 11q22-23 deletion, a 17 q deletion trisomy 12) associated with a poor clinical outcome whereas patients with mutated CLL cells have chromosomal changes (e.g 13q14 deletion) associated with better survival. There are five major chromosomal abnormalities observed in CLL: deletion 13q, trisomy 12, deletions 11q, 17p and 6q (Zenz *et al*, 2007).

2.9.1. Chromosome 13 anomalies

Chromosome 13 abnormalities in CLL are characterized by a deletion of band 13q14 or deletion of a larger region of the chromosome. Deletions on chromosome 13 are the most common genetic abnormality in CLL, occurring in approximately half of all CLL cases (Hamblin, 2007). Deletions in the long arm of chromosome 13 typically occur at 13q14.3 in a region containing the retinoblastoma gene *RB1* (Inamdar & Bueso-Ramos, 2007). Several other genes (*DLEU1*, *DLEU2*, *RFP2*, *KCNRG*, *DLEU6*, *DLEU7* and *DLEU8*) and microRNAs (*miR15* and *miR16* (Calin *et al*, 2002)) are also present in this chromosomal region.

2.9.2 Trisomy 12

Trisomy 12, characterized by an extra copy of chromosome 12, represents the next commonest abnormality occurring in between 10 and 20% of cases (Inamdar & Bueso-Ramos, 2007). Ten to twenty percent of all patients have CLL cells with trisomy 12 either as the only genetic defect or in combination with other chromosomal abnormalities (Acar & Connor, 1998). Interestingly, CLL patients with trisomy 12 have higher expression of CD38, CD25 and/or CD79b when compared to "average" leukaemic cells, even if the genes encoding these cell surface markers are not located on chromosome 12 (Goorha *et al*, 2004;Hjalmar *et al*, 2002;Schlette *et al*, 2003). Trisomy 12 may not be detectable at diagnosis but is more commonly seen in the leukaemic cells of patients with advanced disease or Richter transformation (Tsimberidou & Keating, 2005).

2.9.3. Chromosome 11 anomalies

Structural aberrations of chromosome 11, occur in approximately 12 to 25 % of CLL cases (Inamdar & Bueso-Ramos, 2007). The region most frequently deleted in CLL involves the

long arm of chromosome 11 between q14-24 but more particularly bands q22 and q23 (Doneda *et al*, 2003). This is also the region where the mutation of gene ataxia telangiectasia (*ATM*) is found; this gene is involved in the activation of tumour suppressor gene p53 which induces cell cycle arrest and DNA repair or cell death. This mutation is more often found in CLL patients with relatively aggressive disease that is resistant to many therapies (Bullrich *et al*, 1999;Eclache *et al*, 2004). The leukemic cells of such patients may express lower levels of surface CD11a/CD18, CD11c/CD18, CD31, CD48 and CD58 than CLL cells from patients without deletions in chromosome 11 (Dohner *et al*, 1997;Sembries *et al*, 1999).

2.9.4. Anomalies of chromosome 17 at band p13

Ten percent of all patients have deletions in the short arm of chromosome 17 at band 17p13.1 (Inamdar & Bueso-Ramos, 2007). The gene in the region that is typically deleted is *TP53* encoding the p53 tumour suppressor protein. P53 is a 53-kDa nuclear phosphoprotein that plays an important role in the induction of proteins responsible for cell cycle arrest and apoptosis after DNA damages (Sturm *et al*, 2003). CLL patients with *TP53* deletions exhibit higher cell proliferation rates and are characterized by shorter survival and greater resistance to first line therapy (Byrd *et al*, 2004;Cordone *et al*, 1998;Shaw & Kronberger, 2000).

2.9.5. Chromosome 6 anomalies

This chromosomal abnormality involves deletions at the short arm of chromosome 6 (6 q23, 6q25-27 and/or 6 q21) (Fink *et al*, 2005). CLL cells with deletions between region 6q21 and 6q24 generally have higher expression of $CD38^{+}$ and are associated with poor prognosis and short survival (Cuneo *et al*, 2004)(Cuneo *et al*, 2000).

2.10 Cell biology

Chronic lymphocytic leukaemia is a disease characterized by accumulation of abnormal B lymphocytes that appear to be arrested in early G0/G1 phase of the cell cycle and that respond poorly to polyclonal activators (O'Brien *et al*, 1995;Dighiero & Hamblin, 2008). CLL cells have shorter telomeres compared to aged-matched B cells, indicating that these leukaemic cells have prolonged proliferative history (Damle *et al*, 2004). Differences are also found between two subgroups of B CLL cells; telomere lengths of unmutated B CLL cases are much shorter than those of mutated cases, suggesting that unmutated leukaemic cells have a more extensive history of cell division than mutated cases (Damle *et al*, 2004;Chiorazzi & Ferrarini, 2003).

CLL cells are relatively resistant to apoptosis due to overexpression of BCL2, Mcl-1 , Bag-1 and low amounts of pro-apoptotic proteins such as Bax (Schimmer *et al*, 2003;Kitada *et al*, 1998;Gottardi *et al*, 1996). Upon BCR activation, CLL cells escape or undergo apoptosis through IgM or IgD signalling, respectively (Zupo *et al*, 2002;Bernal *et al*, 2001;Zupo *et al*, 2000). These cells are also protected from apoptosis if cultured with other cell types (e.g stromal cells) (Lagneaux *et al*, 1998) or chemokines (Chiorazzi & Ferrarini, 2003). Interaction of CLL-B cells with accessory cells (stromal cells, nurse-like cells) in the bone marrow or lymph nodes is a crucial step for their survival (Munk, I & Reed, 2004). In peripheral blood, a small proportion of mononuclear cells can differentiate into Nurse-like cells (NLCs) that attract CLL cells by expressing chemokines such as stromal derived factor (SDF-1) (Burger & Kipps, 2002). CLL have receptors (CXCR4) for such chemokines and their binding to SDF-1 triggers CLL cells to activate p44/42 MAPK promoting cell survival (Keating *et al*, 2003).

In germinal centres of lymph nodes and in bone marrow, B-CLL cells expressing CD40 can be rescued from apoptosis after ligation with CD154 (CD40 ligand) on activated $CD4^+$ T lymphocytes (Granziero *et al*, 2001).

In addition under the stimulation of CD40, survivin prevents the onset of apoptosis in CLL cells. In the bone marrow, interaction of integrins (CD49d/CD11 or CD11b/CD18) expressed by leukemic lymphocytes with CD54 (ICAM-1) and CD106 of stromal cells induces protection against apoptosis (de la Fuente *et al*, 1999;Plate *et al*, 2000). Protein kinases are critically involved in the regulation of apoptosis and cell survival in CLL cells (Barragan *et al*, 2003). Among these, PKC, PI3K and ERK1/2 are involved in cellular survival while p38-MAPK and JNK have both pro-apoptotic and anti-apoptotic activity. Akt inhibits apoptosis by phosphorylating pro-apoptotic factors such as Bcl-2 and caspase 9 or prevents apoptosis by phosphorylation of the Orphan nuclear receptor TR3/Nur77 (Cardone *et al*, 1998;Pekarsky *et al*, 2001).

3. Quantification of cell dynamics in humans

3.1 Cell Turnover

Cell turnover represents the processes of proliferation and death (or phenotype transition if just referring to one phenotype). Homeostasis, constant numbers of cells within a population, results from a dynamic process where these two key parameters are balanced. The measurement of lymphocyte kinetics is important for the understanding of normal physiological processes, such as the kinetics of immune responses but also disease processes, such as the growth of malignant cell populations (Macallan *et al*, 2003). Cell division and death are important for the pathogenesis of several diseases. In cancer for example, mechanisms controlling proliferation and death rates are perturbed leading to an imbalance of lymphocytes homeostasis. This homeostasis can be affected by different disease states or events which affect pool size. Changes in pool sizes are difficult to measure as the size of a pool is the product of influx into the pool and output from the pool. Therefore, the identification of perturbations in lymphocyte kinetics in diseases such as cancer or immunodeficiency is a key parameter to develop a new therapeutic strategy.

In this chapter, we review the mechanisms of cell division and apoptosis as well as the isotopic labelling technique using deuterium glucose to quantify cell turnover in CLL.

3.2 Cell proliferation

3.2.1 Cell cycle

The cell cycle is a highly ordered process consisting of four phases: G1 (Gap 1 or Growth phase 1), S (DNA synthesis phase), G2 (gap 2 or Growth phase 2) and mitosis (M) (Israels & Israels, 2000) (Figure I.6). After mitosis, cells can enter into quiescence (G0 phase). The phases separating two mitoses constitute the interphase (Meijer L, 2003). Protein synthesis in G1, which increases the cell size to a given threshold, is followed by DNA synthesis in S. During this period, the cell duplicates its genetic material (DNA replication) and a copy of each one of its chromosomes is made. The cell enters in the next phase (G2 phase) in which checks that the replication of DNA has been well performed (post-replicative repair); significant protein synthesis also occurs during this phase, mainly involving the production of microtubules which are required during the process of mitosis. During mitosis, chromosomes are separated and the cell divides into two daughter cells carrying identical chromosomal copies.

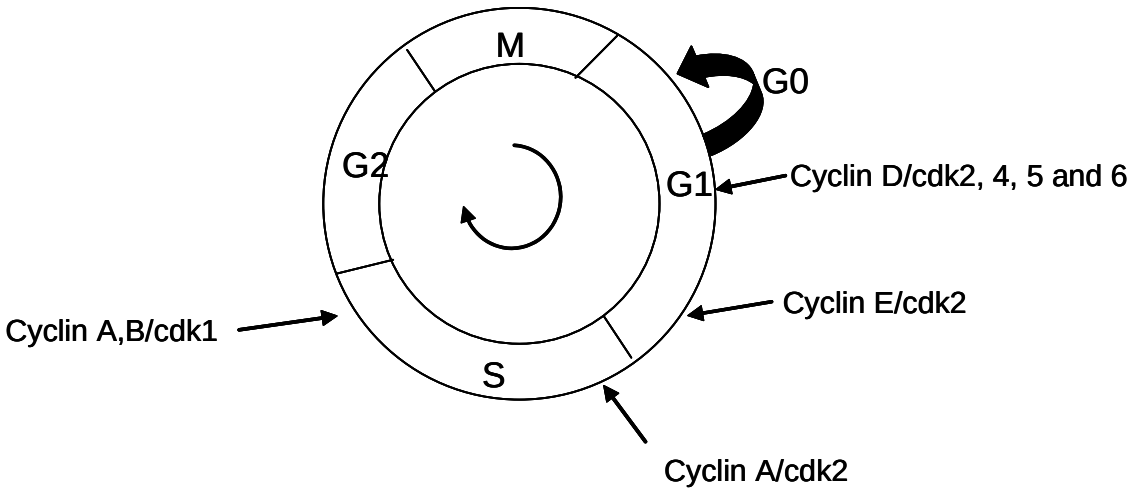

Figure I.6: Scheme representing the phases of the cell cycle (according to (Meijer L, 2003)).

3.2.2 Cell cycle regulation

In eukaryotes, it is imperative that the phases of the cell cycle are precisely coordinated. These different phases must follow a correct order and a phase must be completed before the next one starts, otherwise chromosomes or parts of chromosomes may be lost. Unequal distribution of chromosomes between the two daughter cells may be observed in cancer (aneuploidy). In mammalians, the transitions between cell cycle phases are tightly regulated by cyclins and cyclin-dependent kinases (cdk).

During the G1 phase, cyclin D binds cdk2, 4, 5 and 6 before cyclin E interacts with cdk 2 (Matsushime *et al*, 1992;Xiong *et al*, 1992;Meyerson & Harlow, 1994) (Figure I.6). Following these interactions, the cell cycle progresses from G1 to S phases where cyclin A binds and activates cdk2 (Figure I.6). Cyclin B is synthesized during the S phase, increases throughout G2 phase till mitosis and decreases after anaphase. During G2 phase, cyclin A and B are associated with the cyclin dependent kinase 1 (cdk1) (Figure I.6).

3.2.3 Cell cycle checkpoints

Growth factors are involved in cell cycle initiation (G1-S transition), whereas others (such as TGF-β; Transforming Growth Factor β) increase the length of the G1 phase. Occurrence of mutations/deletions in the DNA or misallocation of chromosomes between the daughter cell is controlled by three checkpoints operating at G1-S, S-G2 and metaphase. The protein p53 plays an important function in cell cycle control. DNA damage leads to the activation of p53 which, once phosphorylated, can induce the arrest of cell cycle during transition between G1-S or G2-M phases by activating genes such as *p2*1 and *14.3.3σ* (Li *et al*, 1994). p21 is a cyclin-dependent kinase inhibitor that binds to cyclin D-cdk4, cyclin A-cdk2 and cyclin E/cdk2 complexes (Xiong *et al*, 1993), which phosphorylate the retinoblastoma protein (Rb) and promote release of transcription factor E2F and G1-S transition. Moreover, the protein

14.3.3σ inhibits the cyclin B-cdk1 complex responsible of the transition from phase G2 to phase M. The cycle arrest caused by these inhibitors gives time for the cell to repair DNA damage before entry into S or M. In the case of irreversible problems, p53 may also induce apoptosis by activating transcription of pro-apoptotic genes such as *Bax* or by suppressing the expression of anti-apoptotic genes such as *Bcl-2*.

3.3 Cell death

Besides apoptosis, different mechanisms of cell death and senescence co-exist including necrosis, autophagy and mitotic catastrophe (Ricci & Zong, 2006).

3.3.1 Apoptosis

Programmed cell death is a physiological response of cells to specific signals of death or lack of survival signals. This process is characterized by morphological and biochemical changes including; cell shrinkage, changes in the cytoskeleton, release of mitochondrial cytochrome c, condensation of chromatin and cleavage of DNA into small fragments (Zimmermann *et al*, 2001). In addition, phosphatidylserine appears at the cell surface, allowing recognition of apoptotic cells by phagocytic cells such as macrophages and dendritic cells. The process of apoptosis is important because it ensures a correct development of embryonic tissue, such as organization of the nervous system, formation of fingers in the foetus and regulation of the immune response. Two distinct apoptotic pathways are recognised, the intrinsic involving mitochondria and the extrinsic mediated through receptor binding. These pathways are controlled by proteolytic enzymes called caspases acting as initiators (caspase -2, -8, -9 and -10) or effectors (caspase-3, -6 and -7) (Zimmermann *et al*, 2001).

3.3.1.1 Intrinsic pathway

This pathway involves the Bcl-2 family of proteins (Hockenbery *et al*, 1990) which includes twenty members, divided into three categories according to the presence of Bcl-2 homology domains (BH1, BH2, BH3 and BH4) and according to their pro- or anti-apoptotic activity.

- Anti-apoptotic members contain at least BH1 and BH2 domains or 4 domains as in Bcl-2, Bcl-Xl, Bcl-w and Mcl-1.
- Pro-apoptotic Bax, Bak and Bok contain BH1, BH2 and BH3 but not BH4.
- Bid, Bad, Bim, Blk and HRK are pro-apoptotic members containing only a BH3 domain.

Following a variety of extracellular and intracellular stresses, pro-apoptotic factors undergo a conformational change leading to the exposure of the BH3 domain and translocation to the mitochondria. This process leads to the release of cytochrome c into the cytosol from the mitochondrial intermembrane space. Cytochrome c then binds to Apaf-1 (apoptotic protease activating factor-1), which recruits procaspase-9 (inactive form of caspase 9) forming the apoptosome. The apoptosome can recruit procaspase-3 that is cleaved by caspase-9, thereby releasing activated caspase-3 and mediating apoptosis (Li *et al*, 1997). Another protein involved in the intrinsic pathway called Smac/DIABLO is also released from mitochondria and binds to the IAPs (inhibitor of apoptosis proteins) inducing the activation of caspases (Du *et al*, 2000;Verhagen *et al*, 2000).

3.3.1.2 Extrinsic pathway

The extrinsic pathway involves the death receptors TNFR (Tumor Necrosis Factor receptor), Fas and TRAIL (TNF related apoptosis inducing ligand) which are located in the plasma membrane of the cell. These receptors contain a cytosolic death domain (DD) leading to apoptosis once bound to their ligands (TNF, FasL, TRAIL) (Zimmermann *et al*, 2001). Ligation of Fas by FasL induces a death signal by recruiting a protein FADD (Fas-associated DD) whose N-terminal end contains a death effector domain (DED) which recruits pro-caspase-8. Once activated, caspase-8 triggers apoptosis via caspase-3 (Ashkenazi & Dixit, 1998). In the TNF pathway, ligation of the TNFR death receptor leads to activation of NFκB (Van Antwerp *et al*, 1998). Apoptosis of immune cells (i.e. $CD8^+$ T cells, natural killer cells and dendritic cells) occurs through the extrinsic pathway. Cytotoxic T lymphocytes induce apoptosis via exocytosis of specialized vesicles containing perforin and granzymes.

3.4 Deuterium Glucose: 6,6-$^2H_2$-glucose

3.4.1: Introduction

Cell proliferation can be directly measured using incorporation of labelled nucleosides into DNA (e.g. ^{3}H-thymidine, [^{3}H] dT) or the nucleoside analogue, 5-bromo-2-deoxyuridine, BrdU) (Hellerstein, 1999;Macallan *et al*, 1998). However the use of tracers such as [^{3}H] dT or BrdU have some important limitations. These radioisotopes are toxic and their use is therefore limited in humans (Macallan *et al*, 1998). Since [^{3}H] dT and BrdU are incorporated through the salvage pathway, DNA replication may be underestimated (Chiorazzi, 2007). A fundamental consideration is that the entry and incorporation of extracellular pyrimidine nucleosides into cellular deoxyribonucleotide triphosphate (dNTP) pools via the salvage pathway is unpredictable and variable (Reichard, 1988;Cohen *et al*, 1983). Other approaches

using Ki67 and CFSE are also used to quantify cell proliferation. Ki-67 is a nuclear antigen expressed by proliferating cells throughout the different phases of the cell cycle but not in G0 phase. Ki67 does not require metabolic labelling but does not allow determination of the rate of progression through cell cycle as cells may divide at a site distant to the sampling site. Finally, the labelling of cells with carboxyfluorescein succinimidyl ester (CFSE) is a useful approach to measure cell division but requires removal cells from the organism for the staining with a risk that it is not representative of normal physiology (Busch *et al*, 2007).

To address these limitations, alternative isotopic labelling methods using deuterium (e.g. 6,6-$^{2}H_2$-glucose and $^{2}H_2O$) have been developed to label DNA of dividing cells in humans. In our study, we used deuterium glucose which is labelled with two deuterium atoms linked to the carbon at C6 (Figure I.7). Since deuterium glucose is not a radioisotope, this approach is safe for use in humans. In addition, labelling cells through the *de novo* nucleoside synthesis pathway, as opposed to the nucleoside salvage pathway, has the advantage that during cell proliferation, key enzymes controlling the synthesis of dNTP are upregulated whereas enzymes of the salvage pathway are down-regulated (Reichard, 1978; Reichard, 1988; Busch *et al*, 2007). Thirdly, labelling of purine in the *de novo* nucleoside pathway is more efficient for purines than for pyrimidines.

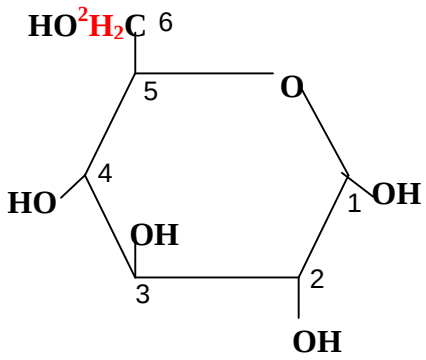

Figure I.7: Structure of glucose labelled with deuterium (6,6-$^{2}H_2$-glucose)

3.4.2: Biosynthetic labelling of DNA using deuterium glucose

As illustrated on Figure I.8, glucose is the principal precursor for nucleotide pentose-ring biosynthesis. Incorporation of deuterium from 6,6-$^{2}H_2$-glucose occurs through the *de novo* nucleoside synthesis pathway. In the cell, deuterium glucose is phosphorylated to glucose-6-P by a *hexokinase*. Most glucose-6-P will enter glycolysis producing ATP but some enters the pentose-phosphate pathway producing ribose-5-P. Glucose 6-P is oxidized to gluconate 6-P and converted to ribulose 5-P (Ru5P) by *gluconate 6-P dehydrogenase* in the presence of $NADP^+$ (Garrett & Grisham, 2000). Ribulose 5-phosphate is then converted to ribose 5-P (R5-P) through the non-oxidative pentose-phosphate pathway by *Ribose 5-phosphate isomerase* (a key enzyme in the pentose phosphate pathway). The ribose 5-P is then converted to phosphoribose pyrophosphate (PRPP) by *Ribose 5-P pyrophosphokinase*. Synthesis of purine nucleotides begins with PRPP and leads firstly to the formation of the nucleotide inosine 5'-monophosphate (IMP) which is converted either to AMP or GMP. The reduction of the 2'-position carbon of purine and pyrimidine ribonucleotides, catalyzed by *ribonuclotide reductase (RR),* generates deoxyribonucleotide diphosphate (dNDP) which, in turn, is phosphorylated to produce deoxyribonucleotide triphosphates (dNTP) (Granner D.K., 2003). Through this pathway, deuterium replaces the non-exchangeable hydrogens at carbon C5' of the deoxyribose moiety within deoxyadenosine 5'-phosphate in newly synthesized DNA (Macallan *et al*, 1998), (Figure I.9).

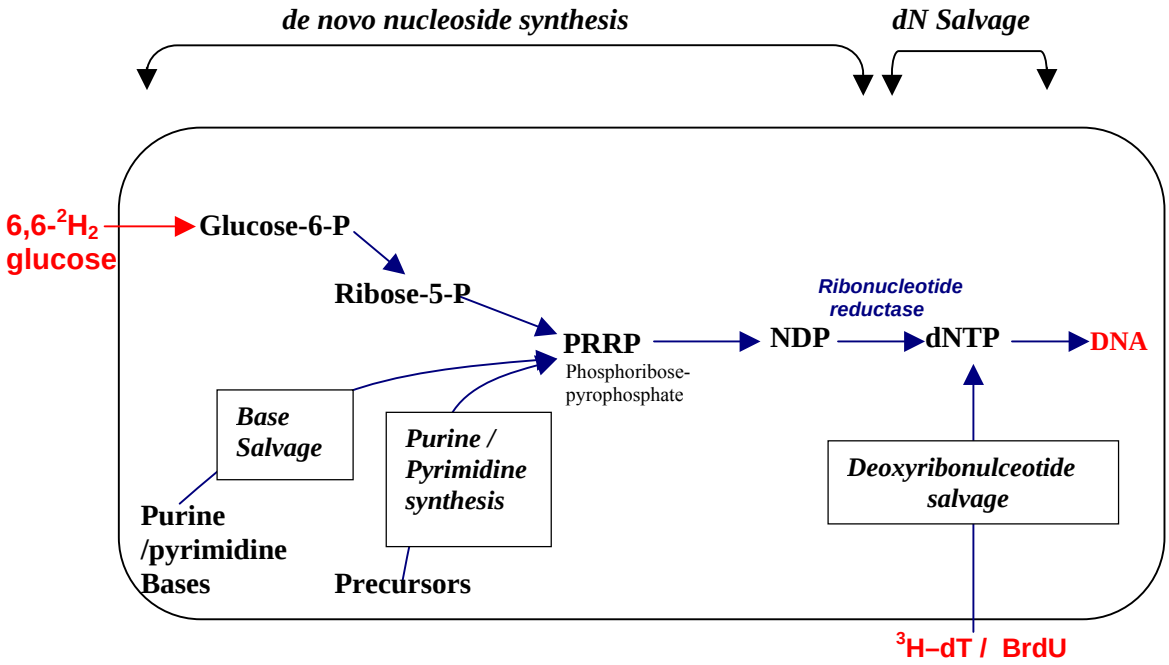

Figure I.8: de novo nucleoside synthesis pathway used by deuterium glucose (6,6-$^{2}H_{2}$- glucose) for measuring DNA synthesis (adapted from (Macallan *et al*, 1998)).

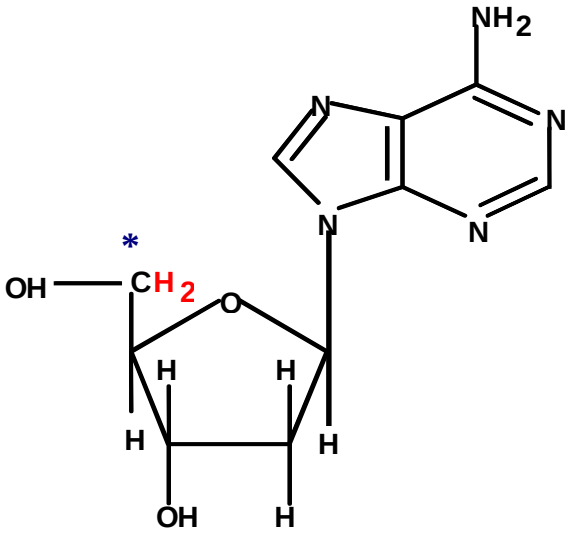

Figure I.9: Structure of deoxyadenosine labelled with deuterium during the *de novo* nucleoside synthesis pathway.

3.4.3: Concepts of the method of labelling using deuterium glucose (6,6-$^2H_2$-glucose)

If a cell divides during the labelling period, deuterium will be incorporated into the DNA. Each of two daughter cells produced will contain one strand of original DNA and one newly synthesized strand containing deuterium labelled deoxyadenosine. If these two daughter cells divide again in the continuing presence of label, six of the eight DNA strands are labelled. Quantitation is achieved by analyzing the amount of label in the DNA of sampled cell populations to determine the amount of deuterium enrichment that occurs during the labelling period. Any cell that proliferates in presence of deuterium glucose will incorporate a certain amount of label into its DNA. This amount is dependent on two factors:

1) The percentage of total plasma glucose that is enriched with deuterium (i.e. % plasma glucose enrichment, the "precursor" enrichment.

2) The dilution factor of deuterium between plasma glucose and DNA of proliferating cells. This dilution factor is relatively constant and has been estimated to be approximately 60-65% (Kovacs *et al*, 2005; Macallan *et al*, 1998).

A cell population with a high rate of turnover will incorporate large amounts of the isotope whereas one with few mitoses will incorporate little. Based on the rate of deuterium uptake, it is possible to measure proliferation and disappearance rates. Proliferation rate is measured according to deuterium incorporation into dividing cells while disappearance rate of labelled cells reflects loss of deuterium from cell pool by death, migration of cells out of peripheral blood or change of cell phenotype. Details of measurements and analyses of cell turnover in CLL are described in the article of British Journal of Haematology in chapter 1 of the result section.

3.4.4: Mathematical Modelling

Quantitative understanding of immunology requires the development of experimental and mathematical techniques. The kinetics of label incorporation and loss are analysed using a mathematical model to lymphocyte labelling data. Model-free method has major disadvantages compared with a modelling approach:

1) Preferential time points: Use of the peak of labelling as a surrogate for cell proliferation means that only a single time point contributes to the measurement of proliferation. This has two disadvantages; firstly it is very sensitive to experimental error and secondly the answer obtained may depend on the time point chosen.

2) Comparability: The expression of proliferation and disappearance in standardized units (% of cells proliferating in a day, % of cells disappearing in a day) enables comparison of results between experiments and between different methods of quantifying lymphocyte kinetics. This is impossible with a model-free approach that measures peak height of deuterium enrichment.

3) Hidden assumptions. The model-free approach assumes as the modelling approach that a cell becomes labelled upon division. However the model-free approach makes two further assumptions: that label increases linearly during the labelling period and that labelled cells do not die before the peak of label. These assumptions could lead to inaccuracies in data interpretation.

3.5 Measurement of B-cell turnover in CLL

As mentioned above, the maintenance of stable B lymphocytes numbers results from a complex equilibrium between cell proliferation and cell death. A modification of one, or both, of these parameters might thus potentially lead to the accumulation of cells observed in CLL. Studies using radioactive approaches to measure turnover rate *in vivo* have indicated that CLL cells proliferate (Dormer *et al*, 1983;Dutcher, 1984;Schiffer *et al*, 1969;Stryckmans *et al*, 1969;Stryckmans *et al*, 1977;Theml *et al*, 1977;Theml & Ziegler-Heitbrock, 1984;Zimmerman *et al*, 1968). In fact, labelling with ^{3}H-thymidine in 2 CLL patients has estimated the fraction of newly formed lymphocytes appearing in the blood to be about 0.5%/day (Theml *et al*, 1977). Different labelling approaches such as using chromium 51 or indium 111, or extracorporeal irradiation of blood have confirmed that CLL cells do proliferate (Zimmerman *et al*, 1968;Schiffer *et al*, 1969;Dutcher, 1984). However, since CLL cells are unresponsive to mitogens (i.e phytohemagglutinin) *in vitro*, it was concluded that they were likely to be unable to proliferate *in vivo*. CLL was therefore considered to be a disease of accumulation due to a defect of apoptosis and not to an excess of proliferation (Dameshek, 1967). This dogma has remained the main hypothesis for four decades in explaining abnormal B lymphocytes accumulation in CLL. Recently, *in vivo* heavy water ($2H_2O$) labelling of 19 CLL patients suggested that CLL is not a static disease, contrasting with Dameshek's dogma (Messmer *et al*, 2005). CLL is thus a dynamic process composed of cells that proliferate and die at detectable levels. These analyses demonstrated that proliferation rates varied from 0.1%/day to greater than 1%/day, with a death rate ranging from – 0.33%/day to 2.14%/day. Patients characterized by a proliferation rates greater than 0.35 per day were much more likely to develop progressive disease than those with lower proliferation rates.

3.6 The cell turnover as a prognostic marker

CD38 is a potential bad prognosis marker in CLL. In Calissano's study, twelve CLL patients were labelled with heavy water for 6-8 weeks. It appeared that $CD19^+/CD5^+/CD38^+$ cells incorporate significantly more deuterium than their $CD38^-$ counterparts during the labelling period (0.92%/day v 0.53%/day, P=0.01), suggesting that CLL cells expressing $CD38^+$ divide more rapidly than $CD38^-$ (Calissano *et al*, 2007). It was suggested that accelerated turnover of $CD38^+$ cells represents a marker of poor prognosis of the disease. These conclusions are further supported by the fact that $CD38^+$ cells show higher levels of expression of markers of proliferation (Ki-67) and cellular activation (CD69), as well as higher levels of telomerase (Damle *et al*, 2007).

4 RNA Turnover in vivo using deuterium-labelled glucose

4.1 Introduction

In our previous chapters we have described cell turnover, we will review here RNA turnover and the metabolic pathway of RNA labelling through de novo nucleoside synthesis pathway. There is a link between RNA turnover, RNA content and protein metabolism. Changes in RNA levels occur in response to cytokine or hormone action or cell division itself (Grimble *et al*, 2000). Variations in both the type and amount of RNA in the cell are pivotal for the regulation of intermediary metabolism. Cellular RNA is in a state of rapid turnover and different RNA species have widely varying life-spans. Messenger RNAs (mRNA) have the shortest survival times, with half-lives of minutes to hours (Ross, 1995) and modification of mRNA stability is an important regulatory point for control of protein translation. Transfer RNAs (tRNA) have longer half-lives measured in hours/days, whereas ribosomal RNAs (rRNA) persist for several days. Estimates for the half-life of rRNA in cells *in vitro* are 7.5 ± 1.5 days in cultured rat fibroblasts (Halle *et al*, 1997), <3 days in human fibroblasts (Gillery *et al*, 1995) and 3.8 days for the 18S rRNA moiety in H1299 cells (Yi *et al*, 1999). RNA turnover represents the aggregate rate of synthesis and degradation of all RNA species. Changes in mRNA levels are best addressed using molecular approaches or microarrays. Small RNA species such as tRNA may also be quantified using specific probes. Many studies have correlated dysregulation of ribosome biosynthesis with cancer, showing that over-expression of ribosomal RNA (rRNA) could lead to excessive protein synthesis and be involved in malignant transformation (Ruggero & Pandolfi, 2003). Radiolabelled U-C^{14}-labelled glucose or cytidylic acid and ^{3}H-uridine have been used to estimate the turnover of RNA in rat organs (GERBER *et al*, 1960) and *in vitro* (Cooper, 1972). Similar approaches are however limited in clinical trials due to the toxicity of such radioactive isotopes.

4.2 Biosynthetic labelling of RNA using deuterium glucose (6,6-$^2H_2$-glucose)

Since accurate methods have failed to measure RNA turnover *in vivo*, we used the safe deuterium glucose approach (Macallan *et al*, 1998). As for DNA, RNA is labelled through the *de novo* nucleoside synthesis pathway. [6,6-$^2H_2$]-glucose is converted by phosphorylation and enters into the pentose cycle (Figure I.10). Ribose 5-P is synthesized and converted in phosphoribosyl pyrophosphate (PRPP) by ribose 5-P pyrophosphokinase (Garrett & Grisham, 2000). Synthesis of purine nucleotides begins with PRPP and leads to first formed nucleotide, inosine 5'-monophosphate (IMP) which is converted either to AMP or GMP. The ribonucleotide diphosphate (NDP) is phosphorylated to ribonucleotide triphosphate (NTP) via nucleoside diphosphate kinases (NDKs). If a deuterium-labelled precursor is used with deuterium at the C6 position, deuterium will replace the hydrogen at carbon C5' of the ribose moiety within adenosine 5'-phosphate in the newly synthesized RNA. From the changes in deuterium content in RNA, it is possible to measure the rate of synthesis of RNA and the rate of loss of labelled RNA, as described in the Results section, chapter 2.

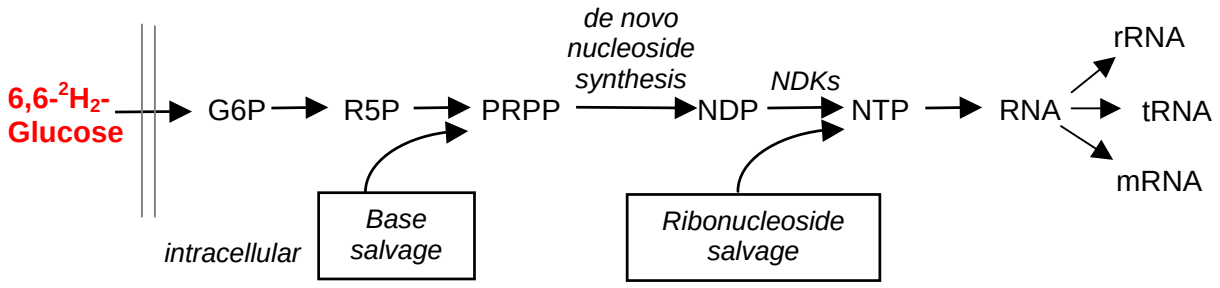

Figure I.10: Metabolic pathway of RNA labelling through de novo nucleoside synthesis pathway

Not all intermediates are shown. G6P, glucose 6-phosphate; R5P, ribose 5-phosphate; PRPP, phosphoribosepyrophosphate; NDP, ribonucleoside diphosphate; NDKs, nucleoside-diphosphate kinases; NTP, ribonucleoside triphosphate.

II. AIMS

The primary aim of this work was to determine the DNA and RNA turnover rates of leukaemic cells in CLL patients. These parameters are important for our understanding of the mechanisms determining cell accumulation and metabolic activity in CLL. By using the safe deuterium glucose labelling technique, the dynamics of DNA and RNA in leukaemic cells in humans may be safely determined.

In the first part of our work, we shall measure the DNA turnover to determine whether CLL is a latent or a proliferative disease. We shall compare proliferation and disappearance rates of B-lymphocytes in 7 CLL patients with 7 healthy controls. After MACS sorting B and T cell populations, DNA will be extracted and hydrolyzed to deoxyribonucleosides. Purified deoxyadenosine will be derivatized and analysed by GC/MS to measure the precise amount of deuterium in the DNA. On the basis of the rates of deuterium uptake and loss, it will be possible, by applying a mathematical model, to determine proliferation and disappearance rates.

Secondly, we shall develop a new method to measure RNA turnover based on incorporation of deuterium glucose into RNA of dividing cells. We hypothesize that RNA will be labelled in the same way as DNA with deuterium-labeled glucose through the *de novo* nucleoside synthesis pathway. We shall adapt the protocol previously described for DNA labelling to analyse deuterium enrichment into RNA. We shall also design a new mathematical model for estimation of synthesis rate of RNA and rate of loss of labelled RNA. We shall investigate RNA turnover in rapidly proliferating tissue culture cells *in vitro* and in one CLL patients to demonstrate that this approach may be applied *in vivo* to measure ribosomal RNA turnover in circulating lymphocytes. Finally, we shall measure RNA turnover in B lymphocytes from CLL and healthy subjects.

III RESULTS & DISCUSSIONS

1. Reduction of B cell turnover in chronic lymphocytic leukaemia

Measurement of cell turnover is important for the understanding of mechanisms involved in accumulation of CLL cells. Previous studies addressing this issue have not given a clear answer (Dormer *et al*, 1983;Dutcher, 1984;Schiffer *et al*, 1969;Stryckmans *et al*, 1969;Stryckmans *et al*, 1977;Theml *et al*, 1977;Theml & Ziegler-Heitbrock, 1984;Zimmerman *et al*, 1968). It was first suggested that CLL lymphocytes are unable to proliferate *in vivo* and thus that CLL is a disease of accumulation of dormant lymphocytes (Dameshek, 1967). However, recent studies using heavy water to measure B cell turnover in CLL have shown that, in contrast, CLL is a disease characterized by a dynamic process of cells that proliferate and die at detectable levels (Messmer *et al*, 2005). However, in this study, CLL B cell turnover was not compared with healthy controls and whether cell proliferation is increased, decreased or unaltered relative to the healthy state was not determined. Macallan *et al* has shown that B cells in healthy subjects labelled by intravenous infusion with deuterium glucose over a period of 24 hours have a proliferation estimated at 1.81 %/day (Macallan *et al*, 2005). These data suggest that normal B cells have higher proliferation rates than CLL cells.

To compare the turnover rates in CLL and normal cells, CLL and healthy subjects were labelled orally with deuterium glucose (6,6-$^{2}H_2$-glucose) over a 10 hour period and proliferation and disappearance rates modelled. B cells and T cells were sorted by MACS and GC/MS analysis was used to quantify deuterium incorporation of these cells. Results are described in the following paper.

bjh research paper

Reduction of B cell turnover in chronic lymphocytic leukaemia

Julien Defoiche,[1]* Christophe Debacq,[2]* Becca Asquith,[3]* Yan Zhang,[4] Arsène Burny,[1] Dominique Bron,[5] Laurence Lagneaux,[5] Derek Macallan[4] and Luc Willems[1]

[1]Molecular and Cellular Biology, FUSAG, Gembloux, [2]GlaxoSmithKline Biologicals, Rixensart, Belgium, [3]Department of Immunology, Imperial College, London, [4]Centre for infection, St George's University of London, London, UK, and [5]Department of Experimental Haematology Bordet Institute, ULB, Brussels, Belgium

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Correspondence: Prof. Luc Willems, Molecular and Cellular Biology, FUSAG, 13 Avenue Maréchal Juin, 5030 Gembloux, Belgium.
E-mail: willems.l@fsagx.ac.be
*These authors contributed equally to this work.

Summary

Whether chronic lymphocytic leukaemia (CLL) is a latent or a proliferating disease has been intensively debated. Whilst the dogma that CLL results from accumulation of dormant lymphocytes is supported by the unresponsiveness of leukaemic cells to antigens and polyclonal activators, recent *in vivo* kinetic measurements indicate that B lymphocytes do divide at significant rates in CLL. However, an important and still unanswered question is whether CLL cells proliferate faster or slower compared with their normal counterparts. This report addressed directly this point and compared B-cell kinetics in CLL subjects and healthy controls, using a pulse-chase approach based on incorporation of deuterium from 6,6-$^2H_2$-glucose into DNA. We confirmed that B cells proliferated at significant levels in CLL but found that the proliferation rates were reduced compared with healthy subjects (mean 0·47 vs. 1·31%/d respectively, $P = 0·007$), equivalent to an extended doubling time of circulating B cells (147 d vs. 53 d). In conclusion, CLL B cells proliferate at reduced levels compared with healthy controls. CLL is thus characterized by an aberrant B-cell kinetics with a decrease in cell turnover, an observation that may impact on elaboration of efficient therapeutic strategies.

Keywords: chronic lymphocytic leukaemia, deuterium glucose, gas chromatography mass spectrometry, B lymphocytes, turnover rate.

Chronic lymphocytic leukaemia (CLL) is a disease characterized by accumulation of monoclonal $CD5^+$ B cells with appearance of small mature lymphocytes. CLL is most common among the elderly but it is also observed in younger individuals (Kipps, 1998; Chiorazzi & Ferrarini, 2003; Klein & Dalla-Favera, 2005). The disease has a highly heterogeneous clinical course with time to progression varying from months to many years. Progressive CLL is marked by an increased accumulation of leukaemic cells within the blood, bone marrow and secondary lymphoid organs, where the lymphocyte doubling time is <1 year. Disease progression correlates with a series of parameters including the mutations status of the immunoglobulin variable heavy chain (*IGVH*) genes, microRNA signature and expression of CD23, CD38 or ZAP-70 (Calin *et al*, 2005; Hamblin, 2005; Pekarsky *et al*, 2005; Stilgenbauer & Dohner, 2005). Patients with mutated *IGVH* genes have a more indolent clinical course and a longer survival than those with an unmutated phenotype. Overexpression of ZAP-70 in CLL cells frequently, but not exclusively, correlates with *IGVH* mutational status and poor prognosis (Del Principe *et al*, 2006). ZAP-70 is associated with enhanced signalling by the cell surface immunoglobulin receptor in CLL cells and ZAP-70 positive cells are more responsive to signals derived from their surrounding environment influencing their proliferation and survival (Richardson *et al*, 2006).

Conceptually, maintenance of stable B-cell numbers results from a complex equilibrium between proliferation and death. A modification of one, or both, of these parameters might thus potentially lead to the accumulation of cells observed in CLL. Several decades ago, early prospective studies using radioactive approaches indicated that CLL cells undergo significant proliferation *in vivo* (Zimmerman *et al*, 1968; Schiffer *et al*, 1969; Stryckmans *et al*, 1969, 1977; Theml *et al*, 1977; Dormer *et al*, 1983; Theml & Ziegler-Heitbrock, 1984). Based on perfusion of ^{3}H-thymidine in two CLL patients, the fraction of newly formed lymphocytes appearing in the blood per day was estimated at about 0·5%; proliferation was also observed in lymph nodes and in the bone marrow (Theml *et al*, 1977). Significant proliferation of CLL leukaemic cells was corroborated by other approaches,

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including infusion of chromium 51-or indium 111-labelled leukaemic cells (Zimmerman *et al*, 1968; Dutcher, 1984) or extracorporeal irradiation of blood (Schiffer *et al*, 1969). These *in vivo* observations apparently contrasted with the behaviour of CLL lymphocytes in short-term tissue culture as an *in vitro* model of the normal immunoproliferative reaction (Dameshek, 1967). It appeared that CLL lymphocytes were largely non-responsive to phytohaemagglutinin and were blocked in their differentiation. It was concluded that the CLL lymphocytes might also be unable to proliferate *in vivo* in response to antigen and would persist in a dormant stage, this dogma, known as the Dameshek dogma, has remained the central hypothesis in explaining lymphocyte accumulation in CLL.

Recent *in vivo* kinetics experiments based on incorporation of deuterium into DNA during heavy water (^2H_2O) consumption have shown that proliferation rates of CLL B cells ranged between 0·1% and 1%/d (Messmer *et al*, 2005 and Table I). This study demonstrated that CLL is not a static disease resulting simply from accumulation of long-lived lymphocytes but rather than it is a dynamic process of cells that proliferate and die often at appreciable levels. However, the proliferation rates in CLL patients were not compared with those observed in healthy subjects. Whether cell proliferation is increased, reduced or unaltered in CLL is thus still an open question. In fact, the mean of B-cell proliferation rates in healthy subjects has been estimated at 1·81%/d using another labelling protocol that consisted of a primed 24 intravenous infusion of deuterium glucose (6,6-$^2H_2$-glucose) (Macallan *et al*, 2005 and Table I). These data thus suggest that CLL is a disease characterized by a reduction of cell proliferation but a firm conclusion cannot be drawn because different techniques were used: i.e. type of label (^2H_2O or 6,6-$^2H_2$-glucose), labelling time (84 d or 24 h), administration route (oral or intravenous) and mathematical model. Therefore, we specifically addressed this important question in this report and compared the proliferation rates under identical experimental conditions.

Table I. B-cell proliferation in chronic lymphocytic leukaemia (CLL) patients and healthy controls.

No.	Age, years	*F*-peak, %/d	Proliferation rate (p), %/d	Doubling time, d
CLL 107	60	0·25	0·23	301
CLL 109	76	0·16	0·11	630
CLL 165	64	0·18	0·14	495
CLL 169	40	0·46	0·49	141
CLL 189	58	0·51	0·81	86
CLL 280	63	0·36	0·22	315
CLL 282	65	0·49	0·39	178
CLL 321	50	0·27	0·29	239
CLL 331	71	0·22	0·24	289
CLL 332	46	0·21	0·24	289
CLL 336	74	0·40	0·41	169
CLL 355	71	0·30	0·45	154
CLL 360	60	0·49	0·48	144
CLL 394	55	0·24	0·18	385
CLL 400	63	0·84	1·76	39
CLL 403	49	0·17	0·39	178
CLL 408	63	0·65	1·09	107
CLL 418	67	0·48	–	–
CLL 472	67	0·38	0·54	128
Mean (SD)	61	0·37 (0·18)	0·47 (0·40)	147 (170)
C02	26	1·75	1·52	46
C03	21	2·47	3·45	20
C04	24	1·57	0·71	97
C05	21	2·38	3·61	19
C06	34	2·98	3·03	23
C07	22	1·47	1·39	50
C08	33	0·89	0·89	78
C10	19	2·39	2·16	32
E01R	77	0·52	0·56	124
E02	85	1·14	1·31	53
E04	85	0·63	0·75	92
E05	70	1·10	0·98	71
E06	65	1·91	2·50	28
E07	79	0·51	0·24	289
E08	77	2·93	4·06	17
Mean (SD)	46	1·64 (0·85)	1·81 (1·24)	38 (69)

Methods

Subjects

Seven subjects with CLL were randomly recruited in Bordet's Hospital (Brussels, Belgium) to take part of the study. Diagnosis and characterization of CLL was made following established criteria (white blood cell counts, percentages of B $CD5^+$ lymphocytes, *IGVH* mutation and expression of CD38 and Zap-70, see Appendix S1). None of these patients had received therapy prior to, or at the time of, the investigation. Controls were drawn from seven healthy subjects who provided informed consent form as leukaemic subjects. The study was approved by the Local Research Ethics committee and all procedures were carried out in accordance with the Declaration of Helsinki. Exclusion criteria were diabetes, severe anaemia, pregnancy, HIV positive, mental disorder and metabolism deficiency.

Measurement of cell turnover

Cell turnover was calculated from the quantitative incorporation of deuterium from deuterated glucose into DNA of dividing cells. Both CLL and healthy subjects were labelled by oral administration of 6,6-$^2H_2$-glucose (1 g/kg; Cambridge Isotopes Ltd, Cambridge, MA, USA) over a 10-h period in half-hourly aliquots, as previously described (Ghattas *et al*, 2005). During the labelling period, diet was restricted to small, low-energy meals and blood samples for measurement of plasma glucose deuterium enrichment were taken approximately 2-h. Blood samples for estimation of deuterium

enrichment in DNA were taken at different days after the start of deuterium glucose infusion.

Cell sorting

Peripheral blood mononuclear cells (PBMC) of CLL subjects were isolated by Ficoll density gradient centrifugation from 10 ml of heparinized blood. Monocytes were eliminated either by CD14/CD56-coupled magnetic beads sorting (MACS) or by plastic adherence for 75 min at 37°C in RPMI medium/5% fetal calf serum. After suspension of depleted PBMC in phosphate-buffered saline containing 0·5% bovine serum albumin, the B and T lymphocytes were subsequently sorted with antibodies coupled to magnetic beads and directed against CD3 or CD19 respectively. All cell populations were purified at levels above 98–99% pellets and were cryopreserved in 'RNA-later' (Ambion, Austin, TX, USA).

Analysis of deuterium enrichment

Analysis of deuterium content in DNA was performed essentially as previously described (Macallan *et al*, 1998). Briefly, DNA from sorted subsets was extracted and digested to yield deoxyadenosine, which was converted to its aldonitrile triacetate derivative prior to analysis by gas chromatography–mass spectrometry (GC-MS ions m/z 198 and 200 by positive chemical ionization in selective ion monitoring; 6890/5973 GC-MS; Agilent Technologies, Bracknell, UK). Plasma glucose enrichment was analysed using the same derivative (m/z 328 and m/z 330) to derive an area under curve for glucose-time, which was taken as the precursor enrichment (*b*, in modelling below). In one subject (CLL01), plasma glucose enrichments were predicted from average glucose pool size and turnover rates from the other CLL subjects.

Mathematical modelling

Results were expressed as the fraction of labelled cells (*F*) present on each day, given by the ratio of the enrichment of label in DNA (*E*) and the precursor enrichment, *b* (mean deuterium enrichment in plasma glucose × 0·65), $F = E/b$, as previously described (Asquith *et al*, 2002; Macallan *et al*, 2003, 2005). The magnitude of the peak value for *F* represents a crude measure of the extent of cellular proliferation, although this does not take into account cell death prior to peak labelling.

The kinetics of label incorporation and loss were analysed by fitting as previously described (Macallan *et al*, 2005) mathematical model to the data. The rate of increase of labelled deoxyadenosine (A*) is given by the product of three factors: the precursor enrichment (*b*), the rate of proliferation of new cells (*p*), and the total amount of deoxyadenosine (A). Thus, the rate of increase of labelled deoxyadenosine is lost by death, migration out of peripheral blood, or change of cell phenotype. This is given by A*d*, where (d*) is the rate of loss of labelled deoxyadenosine.

During the labelling period acquisition and loss of label occur concurrently, such that

$$\frac{dA^*}{dt} = bp\mathrm{A} - \mathrm{A}^*\mathrm{d}^* \qquad t \leq \tau$$

where *t* is time and τ is the length of the labelling period. After the labelling period, only loss of label occurs, given by

$$\frac{dA^*}{dt} = -\mathrm{A}^*\mathrm{d}^* \qquad t>\tau.$$

Since $E = \mathrm{A}^*/\mathrm{A}$ and $F = E/b$, the observed labelled fraction at time *t*, *F(t)*, will depend upon the rate of proliferation during labelling, *p*, and the subsequent loss of labelled cells, *d*, according to the following equations, where τ is the length of the labelling period:

$$F(t) = \frac{p}{d}(1 - \mathrm{e}^{-dt}) \qquad t \leq \tau \qquad \text{During the labelling period}$$

$$F(t) = \frac{p}{d}(1 - \mathrm{e}^{-d\tau})\mathrm{e}^{-\mathrm{d}(t-\tau)} \qquad t>\tau \qquad \text{After the labelling period.}$$

This model was fitted to the experimental data using nonlinear least squares regression (SIGMAPLOT; SPSS, Woking, UK) and the parameters *p* and *d* were estimated.

Results

Deuterium incorporation is reduced in CLL cells compared with healthy controls

The B lymphocyte turnover in CLL and healthy subjects was compared using a single approach based on incorporation of deuterium from 6,6-$^2H_2$-glucose. To this end, seven CLL subjects and seven healthy controls were labelled orally with deuterium glucose over a 10 h period. Blood samples were collected regularly at different times post-labelling, PBMC were isolated by Ficoll density centrifugation, B cells were purified by MACS and deuterium incorporation into DNA was quantified by GC-MS. The fraction of labelled B cells was found in most subjects to peak at days 3–10 and fall thereafter (Fig 1).

Peak enrichments, expressed as the fraction of labelled cells per day, ranged from 0·32 to 0·73%/d in CLL patients and from 0·54 to 2·73%/d in healthy controls (Table II). The mean of peak enrichments were statistically different between CLL and control samples (0·53 and 1·33%/d respectively; $P < 0·05$ according to Wilcoxon–Mann–Whitney two-tailed test).

B-cell proliferation is significantly reduced in CLL

Data from CLL and healthy subjects were analysed to derive proliferation (*p*) and disappearance rates (*d*) of B lymphocytes using a model previously described for the analysis of lymphocyte kinetics (Asquith *et al*, 2002; Macallan *et al*,

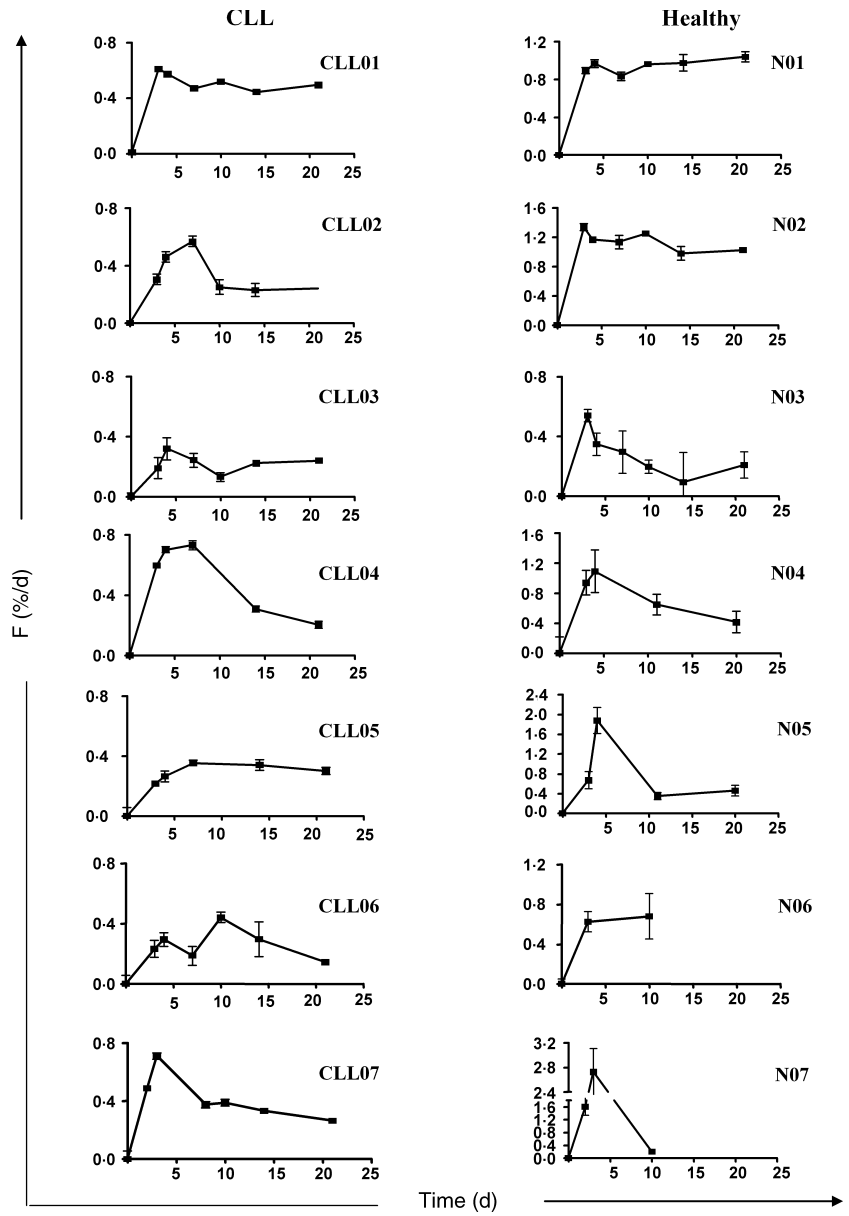

Fig 1. Deuterium enrichment curves of chronic lymphocytic leukaemia (CLL) and control B cells. The cell turnover of seven CLL and seven healthy subjects was measured following oral administration of 6,6-$^2H_2$-glucose over a 10-h period. Blood samples for estimation of deuterium enrichment in DNA were taken at different days post-labelling. Results are expressed as fraction of labelled cells normalized to 1 d of labelling (%/d). The error bars represent standard deviations of triplicate measurements by gas chromatography/mass spectrometry.

2003, 2005) (Table II). As expected from the peak enrichments, the mean proliferation rate was substantially lower in CLL B lymphocytes compared with healthy controls (mean 0·47%/d vs. 1·31%/d, $P = 0·007$, Wilcoxon–Mann–Whitney two-tailed test). This equates to a doubling time for the circulating B-cell pool of about 5 months (i.e. 147 d) in CLL patients compared with 53 d in healthy controls (Table II).

The disappearance rates of the labelled B lymphocyte population refer to recently divided cells and were also estimated according to the same model (Asquith *et al*, 2002). Mean disappearance rates for CLL and healthy subjects ranged from 2·29 to 5·72%/d, equivalent to a half-life of 30 and 12 d respectively (Table II). Although the disappearance rate tended to be lower in CLL than in controls, this reduction was not statistically significantly different ($P = 0·456$, Wilcoxon–Mann–Whitney two-tailed test).

Total B-cell production rates

Although proportional proliferation rates of B cells in CLL are considerably lower than normal values, the B-cell population is much larger. When total productions rates were calculated as the product of the estimated size of the circulating B-cell pool (cell concentration × blood volume) and its turnover rate (p),

Table II. Kinetic parameters of chronic lymphocytic leukaemia (CLL) and control subjects.

			Proliferation				Disappearance	
Subjects	Ages, years	B-cell no. $\times 10^9$ cells/l	Peak fraction of labelled cells, %/d	Proliferation rate (p), %/d	Doubling time (T_2), d	Total production rate (P)§, $\times 10^9$ cells/l	Disappearance rate of labelled cells (d)¶, %/d	Half-life of labelled cells ($T_{1/2}$), d
CLL patients								
CLL01	44	144·5	0·61	0·58	119	3·42	1·25	55
CLL02	67	35·1	0·57	0·41	167	0·83	1·58	44
CLL03	69	99·7	0·32	0·24	289	1·35	0·69	100
CLL04	64	38·4	0·73	0·80	87	0·60	5·08	14
CLL05	65	89·8	0·35	0·29	238	1·23	−0·14	–
CLL06	75	13·1	0·44	0·34	204	0·23	3·01	23
CLL07	49	42·8	0·71	0·63	110	1·28	4·58	15
CLL Mean	62	66·2	0·53	0·47	147†	1·42	2·29	30‡
SD		46·4	0·17	0·21	74	0·99	1·98	33
Control subjects								
N01	51	0·28	1·04	0·88	79	0·012	−0·79	–
N02	44	0·07	1·34	1·31	53	0·005	1·36	51
N03	45	0·08	0·54	0·60	116	0·002	10·03	7
N04	34	0·25¶	1·09	1·20	58	0·015	5·38	13
N05	38	0·25¶	1·88	1·53	45	0·019	7·91	9
N06	29	0·25¶	0·68	0·60	116	0·008	−1·22	–
N07	29	0·25¶	2·73	3·02	23	0·038	17·34	4
N Mean	39	0·20**	1·33	1·31	53†	0·01**	5·72	12‡
SD		0·09**	0·76	0·83	35	0·01**	6·68	19
		*	*	*	*	*	NS	NS

NS, $P = 0{\cdot}456$ according to Wilcoxon–Mann–Whitney two-tailed test between corresponding CLL and control parameters.

*$P < 0{\cdot}05$ according to Wilcoxon–Mann–Whitney two-tailed test between corresponding CLL and control parameters.

†Mean doubling time $T_2 = \ln 2/(\text{mean } p/100)$ was calculated from the mean value of proliferation rate p.

‡Mean half-life time $T_{1/2} = \ln 2/(\text{mean } d/100)$ was calculated from the mean value of disappearance rate d.

§Total production rate = B-cell number × $p/100$ × blood volume.

¶B-cell number in healthy subjects estimated in healthy subjects (5% of B cells from 5 l of blood).

**$n = 7$ (control subjects); absolute B-cell number for seven subjects.

rates for subjects with CLL exceeded control rates by about two orders of magnitude ($1{\cdot}42 \times 10^9$ vs. 10×10^6 cells/d; Table II). These values are consistent with previous reported rates using other methods (Stryckmans *et al*, 1969; Messmer *et al*, 2005), providing further validation for the different experimental approaches and conclusions.

The difference in B-cell kinetics is not attributable to ageing

The subjects with CLL in this study had a mean age of 62 years compared with 39 years in healthy controls (Table II). Although B-cell kinetics remains largely unperturbed by ageing, there may be a slight trend towards reduced B-cell production rates in elderly individuals (Wallace *et al*, 2004). Therefore, we performed comparisons of B-cell turnover with young (mean age 25 years) and elderly (mean age 68 years) subjects (Fig 2). B-cell proliferation was significantly lower in CLL patients compared both with young healthy subjects ($P = 0{\cdot}001$, Wilcoxon–Mann–Whitney two-tailed test) and elderly healthy subjects ($P = 0{\cdot}025$, Wilcoxon–Mann–Whitney two-tailed test). Furthermore, B-cell kinetics were not significantly different between young and elderly healthy subjects ($P = 0{\cdot}101$, Wilcoxon–Mann–Whitney two-tailed test) (Fig 2). We concluded that the slow turnover of CLL B cells was not attributable to ageing.

T-cell proliferation is not significantly reduced in CLL

Following labelling with 6,6-$^2H_2$-glucose, enrichment of deuterium in DNA of $CD3^+$ cells was also analysed in healthy individuals and CLL subjects ($n = 7$). The mean T-cell proliferation rate in CLL was 1·77%/d, equivalent to a doubling time of cells within this population of about 39 d (Fig 3). In healthy individuals, mean of proliferation for the T-cell pool was 1·66%/d, equivalent to a doubling time of 42 d (Fig 3). In contrast to the B lymphocytes turnover, proliferation rates of T cells in CLL were therefore very similar to control values. There was no significant difference in proliferation rates between CLL patients and controls ($P = 0{\cdot}456$, Wilcoxon–Mann–Whitney two-tailed test). Based on these results, we concluded that T-cell turnover is unaltered in CLL subjects.

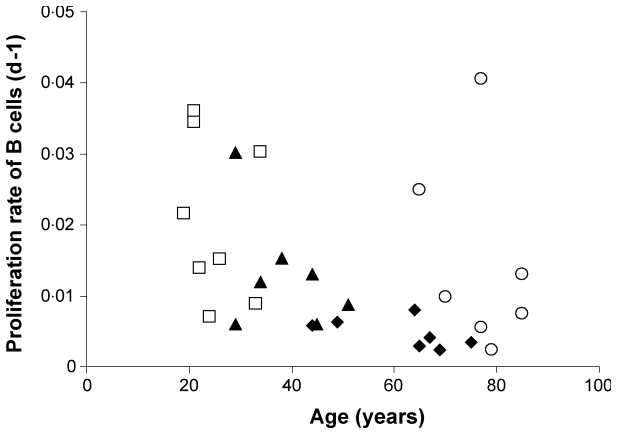

Fig 2. Comparison of B lymphocyte proliferation rates in patients with chronic lymphocytic leukaemia, young and elderly subjects. B lymphocyte proliferation rates in young individuals (□), elderly individuals (○), chronic lymphocytic patients (◆) and N01-7 subjects (▲) according to the subject's age.

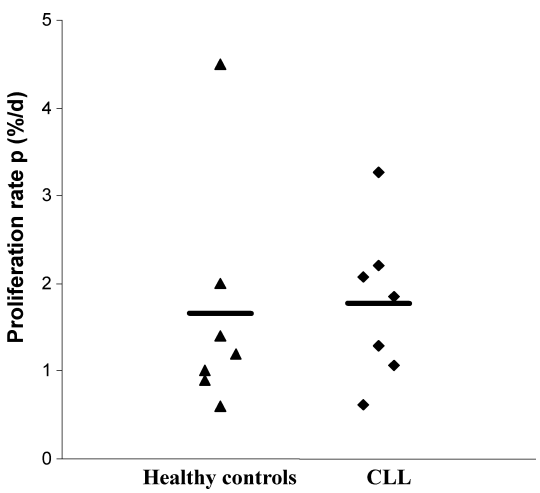

Fig 3. Comparison of T lymphocytes proliferation rates in patients with chronic lymphocytic leukaemia (CLL) and in healthy controls. Results shown are the proliferation rates of T lymphocytes in CLL subjects (◆) and healthy controls (▲) labelled with 6,6-$^2H_2$-glucose.

Discussion

This study compared the turnover rates of the CLL cell population to the kinetic parameters of normal B lymphocytes using a single standardised protocol. We have shown that the daily proliferation rate is significantly reduced in leukaemic lymphocytes compared with healthy controls (0·47%/d vs. 1·31%/d respectively $P = 0·007$, Table II). Consequently, the doubling time of CLL cells is extended by a factor of approximately threefold (147 d instead of 53 d in controls). Our study extends and reinforces two previous independent reports (Macallan *et al*, 2005; Messmer *et al*, 2005) that suggested, but did not formally prove, that proliferation is reduced in CLL. The mean proliferation rates derived from our

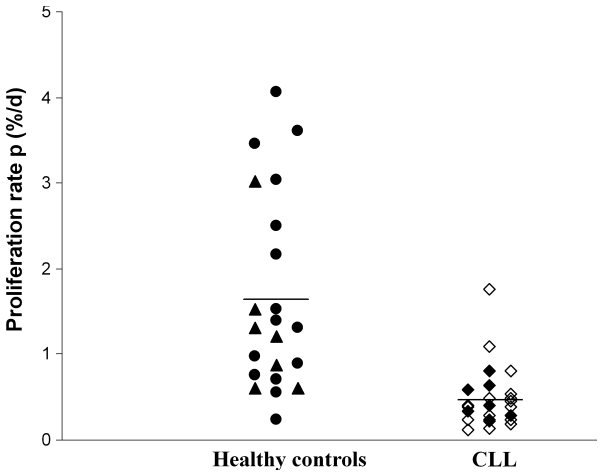

Fig 4. Summary of B lymphocytes proliferation rates in patients with chronic lymphocytic leukaemia (CLL) and in healthy controls. Results shown are the proliferation rates of B lymphocytes in CLL subjects labelled with 6,6-$^2H_2$-glucose (◆, this study) or with ^2H_2O (◇; Messmer *et al*, 2005), healthy subjects labelled orally with 6,6$^2H_2$-glucose (▲, this study) or by intravenous infusion for 24 h (●; Macallan *et al*, 2005).

present study (0·47%/d for CLL and 1·31%/d in healthy controls) are in the range of available data from the literature (0·1–1%/d for CLL (Messmer *et al*, 2005) and 1·5–1·9%/d in healthy controls (Macallan *et al*, 2005), further strengthening and validating all three studies (Fig 4). Interestingly, proliferation rates in indolent CLL [$p = 0·33$%/d (Messmer *et al*, 2005) and $p = 0·47$%/d (this study)] or progressive CLL [0·61%/d; (Messmer *et al*, 2005)] are both below the normal levels [$P = 1·81$%/d (Macallan *et al*, 2005); $p = 1·31$% (this study)]. Together, these data thus clearly demonstrate that B-cell proliferation is reduced in CLL.

The kinetic parameters that we obtained using the deuterium glucose incorporation technique are in accordance with values resulting from other approaches performed several decades ago (Osgood *et al*, 1952; Christensen & Ottensen, 1955; Hamilton, 1956; Fliedner *et al*, 1968; Zimmerman *et al*, 1968; Schiffer *et al*, 1969; Theml *et al*, 1973), although the normal and leukaemic cell populations could not be precisely quantified at that time. Currently, safe measurement of cell proliferation by labelling DNA with stable isotopes can be performed by two main techniques based either on 6,6-$^2H_2$-glucose (Macallan *et al*, 1998) or ^2H_2O (Messmer *et al*, 2005). The latter method has the disadvantage of requiring a very long labelling period with a risk of saturating the dividing population, leading to an underestimate of the proliferation rate of B cells. Conversely, it might be argued that glucose consumption is altered in CLL, for instance, due to excess of cells, leading to a reduction of deuterium incorporation. We think that this possibility is very unlikely because: (i) the 6,6-$^2H_2$-glucose is just a marker within the overall glucose pool, as the patients were not hypoglycaemic; (ii) deuterium enrichment curves were similar in CLL and in the controls

during initial glucose uptake; and (iii) T-cell kinetics was unaltered (Fig 3). Even if the glucose metabolism of CLL and normal cells were different, peak enrichment would not be modified because a very small amount of provided deuterium is incorporated in the DNA backbone during division of the CLL cells (estimated at ±0·001%/d). Finally, the best argument to strengthen the 6,6-$^2H_2$-glucose and ^2H_2O techniques is that both approaches yielded similar proliferation rates.

One drawback of the deuterium incorporation technique used in this report is that sufficient amounts of pure cell populations are required to determine the corresponding dynamic parameters. For example, it was not possible to quantify the turnover of B lymphocytes expressing the CD5 marker in control subjects, because these cells represent only a minor fraction of normal B cells (1–2% of the PBMC of our controls). Another phenotypic marker that would warrant further investigation is CD27, as all CLL cells express this molecule, which normally characterizes the memory subtype (Kipps, 1998). In normal subjects, naïve ($CD27^-$) peripheral blood B lymphocytes divide only slowly (0·46%/d) whereas CD27 positive memory B cells proliferate more rapidly (2·66%/d) (Macallan *et al*, 2005). As these $CD27^+$ B lymphocytes represent only a fraction of all B cells (estimated to about 26–43%), it is likely that the difference in proliferation rates between control and CLL patients in terms of comparable ($CD27^+$) cell populations is even greater than that estimated here taking the whole (CD27-unsorted) B-cell population as a control. It was also not possible to establish a clear correlation between expression of CD38 or ZAP70 and the cell turnover because of the limited number of CLL patients analysed, but this beyond the scope of this report. However, proliferating CLL clones have very recently been shown to preferentially express CD38 and high levels of CD5 (Calissano *et al*, 2007).

Our report adds another level of complexity to the previously proposed model postulating that CLL is a proliferative disease (Messmer *et al*, 2005). Indeed, patients with proliferation rates >0·35%/d are more likely to exhibit active disease. A correlation between proliferation rates and disease progression is currently evaluated as an interesting potential prognostic biomarker for CLL (Murphy *et al*, 2007). In fact, B-cell proliferation rates in the CLL subjects (CLL01 and CLL07) with unmutated IgM were above those in all other subjects except in CLL04. In Messmer *et al* (2005), two CLL patients at Rai stage IV also yielded proliferation rates in the upper range, although not the highest. Both studies thus indicated that there is no strict correlation between proliferation rates and mutational status or clinical status. The working model hypothesizes that leukaemic cells abnormally accumulate because of an excess in proliferation. Our data demonstrate that the imbalance in cell numbers characteristic of CLL correlates with reduced proliferation and a trend to lengthened half-life survival time (Table II). Instead of excess in proliferation compared with normal levels, CLL progression would thus rather be the consequence of increased proliferation occurring below normal levels.

Although proportional proliferation rates of B cells in CLL are considerably lower than normal values, the B lymphocyte population is much larger and the daily production of B cells is impressive: 10^9–10^{10} vs. 10^7 cells/d (Table II and Messmer *et al*, 2005; Theml *et al*, 1973). However, main parameter to consider is not the absolute number of cells but the rate of daily increase in CLL patients, which results from the combination of cell proliferation and death. The present study showed that, although proliferation is reduced in CLL, the absolute cell numbers were stable during the assay, indicating a concomitant reduction in cell disappearance, most probably via a process of apoptosis. Therefore, it appears that the leukaemic B-cell clone is continuously proliferating and dying, although at a reduced rate. Consequently, CLL is a disease characterized neither almost exclusively by a defect of apoptosis nor by an excess of B-cell proliferation. Instead, CLL appears to be the consequence of a subtle imbalance of decreased cell turnover.

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Supporting information

Additional supporting information may be found in the online version of this article:

Appendix SI. Clinical and haematological characteristics of CLL patients.

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Appendix SI: Clinical and hematological characteristics of CLL patients

	Age (a)	Sex (b)	Binet (c)	B cells x 10^9/l (d)	% $CD19^+$ $CD5^+$ (e)	*IgVH* mutation (f)	% $CD19^+$ $CD38^+$ (e)	% $CD19^+$ $CD27^+$ (e)	% $CD19^+$ Zap-70^+ (e)	Splenectomy (g)
CLL 01	44	**F**	**B**	144.5	99	U	32	95	45	-
CLL 02	67	**M**	**A**	35.1	97	M	0.8	91	1	+
CLL 03	69	**M**	**A**	99.7	97	M	8	88	3	-
CLL 04	64	**M**	**A**	38.4	96	M	58	91	1	+
CLL 05	67	**M**	**A**	89.8	94	M	1.3	84	10	-
CLL 06	75	**M**	**A**	13.1	96	M	1	55	11	+
CLL 07	49	**M**	**B**	42.8	81	U	62	58	75	-

(a) in years
(b) female (F), male (M)
(c) Binet stage A and B
(d) B Cells x 10^9/l: B cells in billion per liter of blood
(e) Percentages of double positive cells for the indicated markers were determined by flow cytometry.
(f) DNA sequences at least 2 % different from the most similar germline counterpart were considered mutated (M). U means unmutated.
(g) Spleen was removed in three patients (+).

Cell phenotyping

Whole blood was diluted with PBS to a final concentration of approximately 10^6 cells/100 µl. An aliquot of 100 µl was first surface stained with CD3-FITC and CD19-PC5 (Immunosource) for 15 min at room temperature in the dark and then fixed, permeabilized using the Fix & Perm kit (An Der Grub, Austria) according to the manufacturer's instructions and stained for 15 min with 5 µl of anti-Zap-70-PE (clone 1E7.2) (Immunosource). Cytoplasmic ZAP-70 expression was determined by three-color flow cytometry (CD3/ZAP-70/CD19) and cases were considered as ZAP-70 positive if >20% of CD19+ cells were positive. Expression of ZAP-70 in purified CD19+ was also confirmed by a quantitative reverse transcriptase-polymerase chain reaction. For CD38 labeling, cells were stained with directly conjugated CD38-FITC (Dako A/S, Glostrup, Denmark) and CD19-PE (Immunosource, Halle-Zoersel, Belgium). CD38 antigen expression was analyzed in a CD19+ gate using flow cytometry and was deemed positive if 30% of cells stained positive.

In this work, we have compared B cell turnover in CLL patients with healthy subjects using a approach based on incorporation of deuterium from 6,6-$^2H_2$-glucose. We have first shown that proliferation rate of CLL cells was significantly reduced compared with healthy patients (mean 0.47 versus 1.31 %/day respectively, P=0.007); from this we can deduce that the survival time of CLL cells in the circulation in the peripheral pool is increased from about 53 days to about 147 days. In addition, the comparison of B cell turnover in CLL patients with all control data including our study and previous study (Macallan *et al*, 2005) shows that proliferation rate is significantly reduced (P=0.001 according to Wilcoxon-Mann-Whitney two-tailed test). Moreover the disappearance rate of labelled cells was lower in CLL than in controls but this reduction was not significantly different (P=0.456 Wilcoxon-Mann-Whitney, two tailed test). The total production rate of circulating B cell pool in CLL subjects was increased by 2 orders magnitude (1.42×10^9 versus 10×10^6 cells/day). Finally, we have compared B cells kinetics in young and elderly subjects in order to assess whether B cell kinetics in CLL was perturbed by ageing. Interestingly, we found that the slow turnover of B-CLL cells was not attributable to ageing. We have also demonstrated that T-cell proliferation was no significantly reduced in CLL compared with controls (1.77%/day versus 1.66%/day, P= 0.456 Wilcoxon-Mann-Whitney, two tailed test). Based on these results, we conclude that CLL is characterized by a decreased cell turnover.

2. Measurement of RNA turnover using deuterium glucose

2.1. Introduction

In this part of my project, we aimed to develop an isotopic approach for measuring RNA synthesis rates for use in in vivo studies in human subjects. We hypothesized that RNA could be labelled in the same way as DNA with deuterium-labelled glucose through the *de novo* nucleoside synthesis pathway. The need for changes in reaction conditions and separation protocols was addressed for each stage in the process sequentially, starting with derivatization and Gas Chromatography Mass Spectrometry (GC/MS) and working back up the protocol to cell labelling as the final step. These stages were validated or modified until optimized for RNA/ ribonucleoside analysis using adenosine as a marker of RNA metabolism. Once this protocol was developed, we used this new approach to measure deuterium enrichment in RNA in cell culture *in vitro* and *in vivo* from one CLL patient.

2.2 Materials and methods

2.2.1 Method development

2.2.1.1 Isolation of ribonucleosides from RNA

In order to analyze incorporation of deuterium into RNA, it is necessary to separate ribonucleosides from RNA. RNA (0.1g/ml) was hydrolyzed by sequential digestion with nuclease S1 (18 hours at 37°C), phosphodiesterase I (2 hours at 37°C) and alkaline phosphatase (37°C for 1 hour; all enzymes from Sigma, St Louis, MO), as previously described (Crain, 1990); this protocol is similar to that previously used for DNA (Macallan *et al*, 1998). Efficiency of RNA digestion was confirmed by high performance liquid chromatography (HPLC) using a C-18 column with adenosine, uridine, cytosine and guanosine standards (Sigma, St Louis, MO). Purification of adenosine from other ribonucleosides was achieved using an LC-18 SPE column (Supelco, Bellfonte, PA), adenosine being retained on the column in aqueous phase but eluted by 50% methanol; purity was confirmed by HPLC analysis of aqueous and methanol eluates.

2.2.1.2 Derivatization of ribonucleosides and GCMS analysis

Derivatization conditions required for GC/MS analysis are a compromise between the formation of the derivative and its destruction. For DNA analysis, deoxydenosine may be converted to its aldonitrile triacetate derivative (ATA) by reaction with hydroxylamine/pyridine 1% w/v, at 100°C for 45 min followed by reaction with acetic anhydride at room temperature. Adenosine was derivatized by reaction with the same reagents but at a variety of time and temperature settings as described below. GCMS analysis of the ATA derivatives was performed with a HP-225 column using a 6890/5973 GCMS (Agilent Technologies, Bracknell, UK) under positive chemical ionization with methane, monitoring

ions in SIM at m/z 256 and 258 for adenosine and 198 and 200 for deoxyadenosine, representing the $M_{+0,}$ M_{+2} mass isotopomers of the dominant ion after ionization.

2.2.2 In vitro labelling of PM1 cells in culture

In vitro studies of label incorporation from [6,6-$^2H_2$]-glucose into cellular RNA were performed with PM1 cells (a clone derived from a human cutaneous T-cell lymphoma) grown in suspension (RPMI 1640 media, 10% fetal calf serum, Gibco BRL, Invitrogen UK). Labelled media were made by adding [6,6-$^2H_2$]-glucose (Cambridge Isotope Laboratories, MA, USA) and unlabelled glucose to glucose–free media to achieve a concentration of 0.1g/ml and an isotopic enrichment of 22%. Cells were grown in labelled media for 2, 4, 6, 24 and 48 hours prior to harvesting. RNA was extracted by guanidine thiocyanate / phenol extraction in a mono-phase solution (Trizol Sigma, St Louis, MO); yield and purity were confirmed by spectrophotometry.

2.2.3 In vivo labelling of human lymphocytes

A patient with chronic lymphocytic leukaemia was given 1g/kg 6,6-$^2H_2$-glucose (Cambridge Isotopes, MA, USA) orally over a 10 hour period, as half-hourly aliquots following a priming dose, as previously described (Ghattas *et al*, 2005). This subject was treated for psoriasis (methotrexate) and was a 59 year old man with Binet stage A disease characterized with a peripheral blood B-cell count of 8.9 x10^9 cells/l, and an unmutated *IGVH* phenotype. The study was granted ethical approval by Bordet Hospital in Brussels, Belgium. The subject gave written informed consent and all procedures were performed in conformity with the Declaration of Helsinki. Blood samples were taken for estimation of deuterium enrichment 3, 4, 7, 10, 14, 21 and 30 days after the day of ingestion of labelled glucose. Peripheral blood mononuclear cells **(**PBMC) were isolated by Ficoll density gradient centrifugation (10ml of

heparinized blood) and $CD3^-$ B cells purified by negative selection using CD14/CD56 and CD3-coupled magnetic beads (MACS, Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). After cryopreservation in "*RNA-later*" (Ambion, Austin, Texas USA), both DNA and total RNA were extracted; messenger RNA was isolated from total RNA using MicroPoly(A) Purist kit (Ambion, Austin, Texas USA). Samples were analyzed for deuterium enrichment as described above. Plasma glucose enrichment was also measured using the same derivatization approach (but monitoring ions m/z 328 and 330) as previously described (Macallan *et al*, 1998).

2.2.4 Modelling and Calculation

The kinetics of label incorporation and loss in the *in vivo* studies were analyzed using a mathematical model analogous to that previously described for measurement of DNA/cell turnover (Asquith *et al*, 2002) as shown in Figure III.2.1. Experimental results were expressed as Fractional Enrichment (*F*), defined as the proportion of RNA labelled relative to the labelling rate in its precursor, given by $F= E/b,$ where E is deuterium enrichment in RNA and b is [mean deuterium enrichment in plasma glucose x 0.65], as previously described (Asquith *et al*, 2002;Macallan *et al*, 2003;Macallan *et al*, 2005).

Since enrichment $E = A^*/A$, then $F(t) = A^*(t)/Ab$. Dividing, the equations by Ab, we can express $F(t)$ in terms of the parameters s and l^* :

$$F(t) = \frac{s}{l^*}(1 - e^{-l^* t}) \qquad t \le \tau$$

$$F(t) = \frac{s}{l^*}(1 - e^{-l^* \tau})e^{-l^*(t-\tau)} \qquad t > \tau$$

Measured data was fitted to this model using nonlinear least squares regression (Sigmaplot v8.02; SPSS, Woking, United Kingdom) and solved for best fit values of s and l^* for RNA. Where doubling times (t_2) and half-lives ($t_{½}$) are given, these were calculated as $ln2/s$ and $ln2/l^*$, respectively.

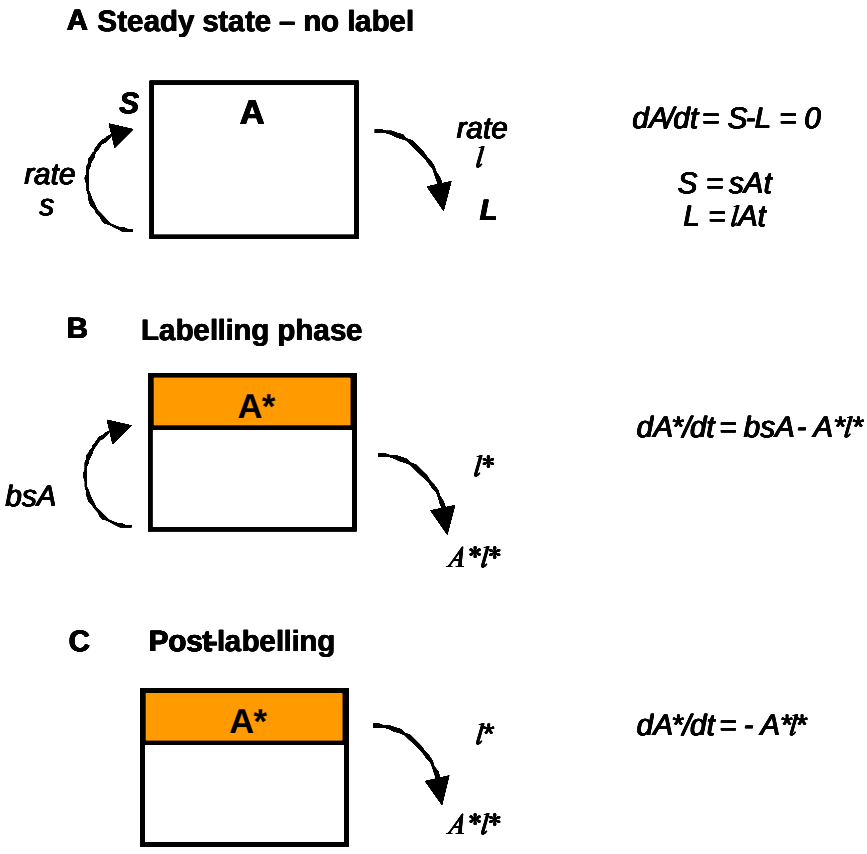

Figure III.2.1: Model for interpretation of RNA labelling in vivo

A. Representation of a pool containing an amount of RNA (A). Over time, t, an amount L of RNA is lost and replaced by an amount of newly synthesized RNA, S. At steady state these amounts are matched, S=L (Figure 2A). Although the rate of production of new RNA is not constrained by the amount of RNA already present, unlike DNA, proportional synthesis rates (the rate of synthesis of new RNA per unit time per unit of existing RNA) can be defined as *s*, where *s* = S/At. The total rate of production of new RNA, S, is then given by S = *s*At. Similarly we may define a proportional rate of loss of RNA per unit time per unit RNA, *l*, where *l* = L/At.

B. During labelling, newly synthesized RNA is produced at rate S/t = sA, of which a proportion, b, will be labelled, where b is the labelling rate of ribonucleoside RNA precursors, which is taken as [0.65 x the labelling rate of glucose]. Thus new labelled RNA is synthesized at rate b*s*A, producing an amount of labelled RNA, A* (the asterix denoting labelled material). At the same time, labelled RNA will be lost. The disappearance rate of labelled material may differ from the average rate for all RNA (*l*) if there is heterogeneity within the RNA pool, so if we designate the rate of loss of labelled RNA as *l**,. The rate of loss of labelled RNA is then given by A**l**.

C. After labelling has stopped, labelled glucose disappears very rapidly so there is no longer any new accrual of labelled RNA. However, loss of labelled material continues at a rate A**l**, proportional to the amount of labelled material remaining. If the length of the labelling period is τ, these equations can be solved to yield:

$$A^*(t) = \frac{bsA}{l^*}(1 - e^{-l^* t}) \qquad t \le \tau \quad \text{during labelling}$$

$$A^*(t) = \frac{bsA}{l^*}(1 - e^{-l^* \tau})e^{-l^*(t-\tau)} \qquad t > \tau \quad \text{post -labelling}$$

2.3 Results

2.3.1 RNA digestion to ribonucleosides and derivatization of adenosine

HPLC confirmed that the enzymatic conditions and SPE column separation protocol described above gave high retrievals of adenosine from RNA with >95% purity. When optimal conditions for derivatization of adenosine were investigated, we observed, firstly, that no appreciable derivatization of adenosine (6mM) occurred at or below 80°C (Figure III.2.2A). However, at the higher temperatures required for effective derivatization, the ATA derivative of adenosine was found to be unstable (Figure III.2.2B). An optimal compromise between production and destruction was found by derivatization at 110°C for 2 hours and this was adopted in subsequent experiments.

In order to compare DNA and RNA turnover in the same cells, an ideal approach would be to derivatize both deoxyadenosine and adenosine concurrently. However optimal conditions for adenosine / RNA (110°C for 2 hours) differ from those for deoxyadenosine / DNA (100°C, 45 minutes). To test whether a compromise set of conditions could be identified, we derivatized equimolar (6mM) concentrations of adenosine and deoxyadenosine together (Figure III.2.2C). At 110°C the derivative of deoxyadenosine is rapidly formed but then degrades whereas the derivative of adenosine only forms more slowly. Although there is a crossover point (about 75 min), under these conditions neither substrate yielded more than about 50% of its maximum yield. We therefore conclude that, unless cellular material is available in abundance, a better approach to obtain maximum yield for each substrate, would be to separate DNA and RNA first and then derivatize separately under different conditions.

To estimate the deuterium content of labelled material, the abundances of the parent ion (m/z 256) and the M_{+2} ion (m/z 258) (Figure III.2.2D) were compared, using area-under-curve values from gas chromatography (Figure III.2.2E). Standard curves generated using 5,5-D_2-ribose (Cambridge Isotopes Ltd, Cambridge, MA) at varying enrichments showed good

correlation between measured and predicted values (Figure III.2.2F). Subsequently standards were run with every set of samples to calibrate results.

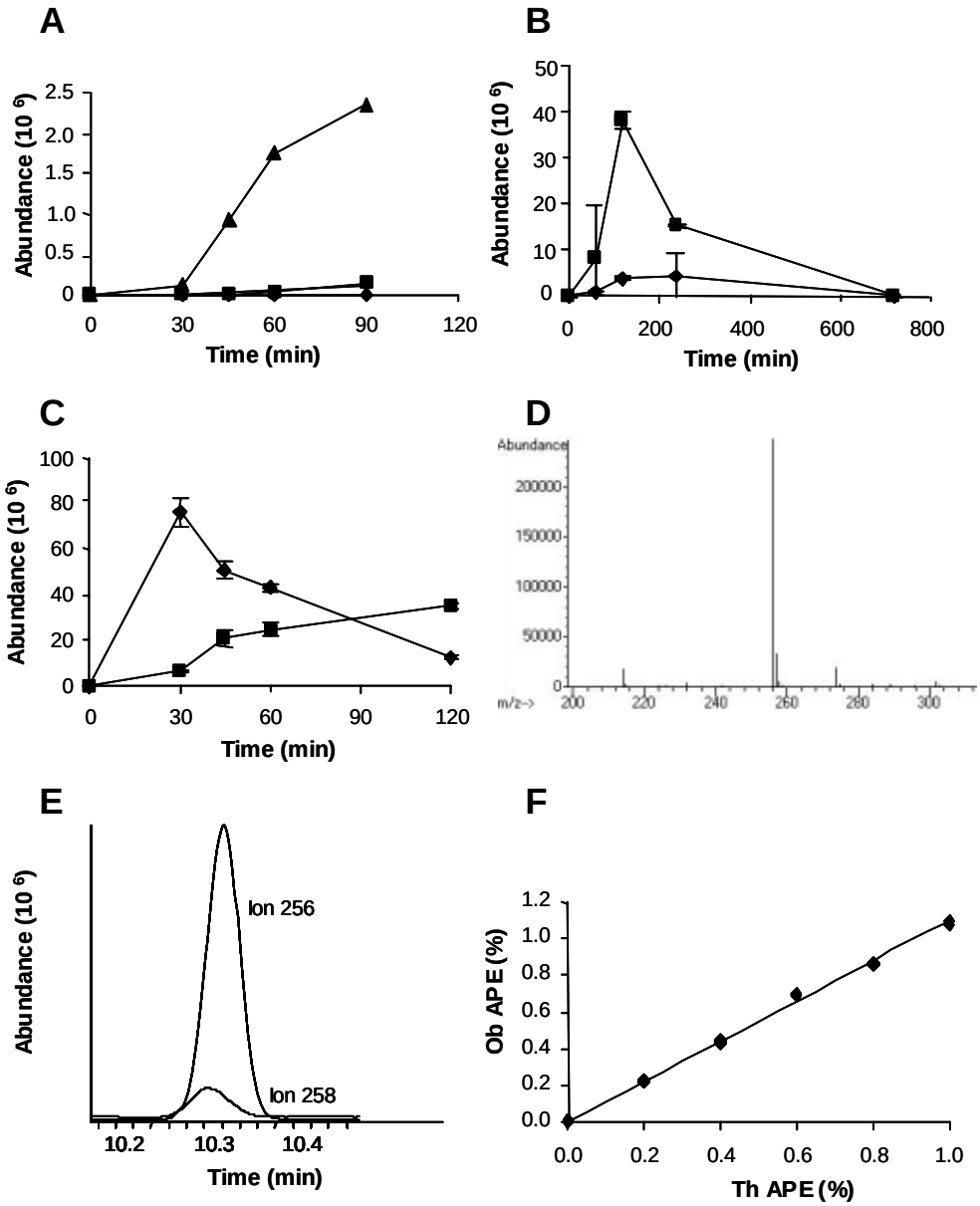

Figure III.2.2: Production of the aldonitrile triacetate (ATA) derivative of adenosine with hydroxylamine/pyridine and acetic anhydride.

Abundance represents the amount of aldonitrile triacetate derivative of adenosine (ATA) formed and is expressed as the GCMS signal in arbitrary units for the relevant ion (m/z 256); (A) Temperature dependence of formation of ATA derivative from adenosine: 60°C (diamond), 80°C (square), 100°C (triangle); (B) Stability of ATA derivative from adenosine at 90°C (square) and 110°C (diamond); (C) Effects of simultaneous derivatization of adenosine (square) and deoxyadenosine (diamond) at 110°C (D) mass spectrum of ATA derivative showing the dominant ion under PCI at m/z 256; (E) Chromatographic curve of the derivative of adenosine unlabelled (m/z 256) and labelled (m/z 258); (F) Observed versus predicted deuterium enrichment for ATA derivative of ribose standards of known enrichment with 5,5-D2-Ribose (0-1%).

2.3.2 Labelling of dividing lymphocytes *in vitro*

When PM1 cells were grown in labelled media, deuterium enrichment progressively increased with time in both adenosine from RNA and deoxyadenosine from DNA. Initially adenosine enriched more quickly than deoxyadenosine (Figure. III.2.3); the fraction of new RNA and DNA were estimated to be 1.9% and 0.9% respectively at 2 hours and 10.3% and 3.7% at 6 hours. As PM1 cells, used in this experiment, are rapidly dividing, a significant component of RNA synthesis might be expected to relate to the production of a new complement of RNA for each new cell. This appeared to be the case beyond 6 hours where the enrichment curves for adenosine and deoxyadenosine (from RNA and DNA respectively) followed parallel slopes (Figure III.2.3). This suggests that almost all measurable RNA production at this stage is related to new cell production, rather than production of new RNA in existing cells; with the latter the curves would continue to diverge. At earlier time points, RNA synthesis exceeded DNA synthesis and we postulate that this is because rapidly turning-over pools of RNA, such as mRNA and tRNA, label rapidly but then saturate beyond about 6 hours.

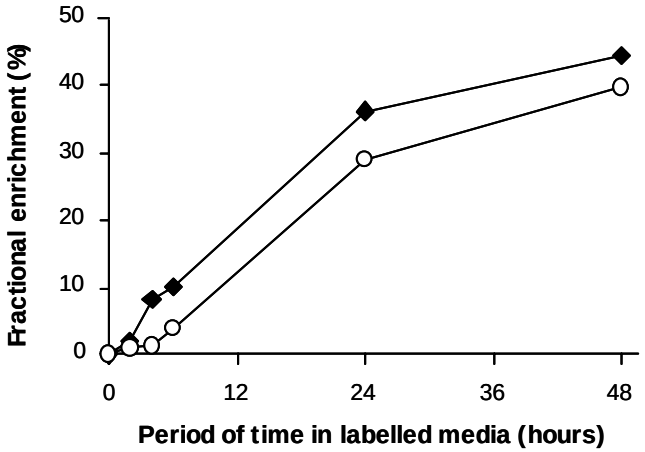

Figure III.2.3. Progressive labelling of RNA and DNA in PM1 cells grown in media labelled with deuterated glucose.

Results are expressed as fractional enrichments (F) of adenosine (filled diamonds) and deoxyadenosine (open circles) which respectively represent the accumulation of newly synthesized RNA and DNA in cells growing in media labelled with 6,6-$^{2}H_{2}$-glucose**.**

2.3.3. In vivo labelling: Turnover of RNA in peripheral blood leucocytes

To investigate *in vivo* production of RNA an analogous approach to that used *in vitro* was utilized but several major differences should be noted: First, labelling *in vivo* was transient, as in a "pulse-chase" experiment, rather than continuous labelling. Secondly, measurements were made in the unlabelling, rather than the labelling phase. Thirdly, as cells *in vivo* had much slower proliferation rates than cells in culture, results are likely to reflect cellular turnover of RNA, as distinct from the production of new RNA in relation to cell division. Fourthly, there was a delay between labelling and measurement; sampling times for PBMC were based on DNA labelling protocols, where sampling is delayed to allow equilibration between vascular and extravascular lymphoid compartments (Macallan *et al*, 2003).

In order to evaluate the impact of such sampling on interpretation, a model was constructed (Figure III.2.4A) estimating the likely relative contributions of mRNA, tRNA, and rRNA to the total labelling curve. From this we deduced that PBMC labelling curves more than 3 days post-labelling reflect almost exclusively changes in rRNA labelling. We've applied this new protocol to measure deuterium incorporation *in vivo* into RNA in peripheral blood $CD3^-$ lymphocytes (predominantly B cells) from one CLL subject.

Peak enrichment expressed as the fraction of labelled RNA per day was found at day 4 post-labelling and then fall thereafter (Figure III.2.4B). Total RNA showed higher initial levels of labelling but label was then more rapidly lost consistent with faster turnover; by day 21, RNA labelling was undetectable. For Total RNA labelling reached levels consistent with a proportional synthesis rate (s) of about 7%/d equivalent to a doubling time (t_2) of 10 days (Figure III.2.4B). Labelled total RNA was lost at a rate (l^*) of about 14.6%/d, equivalent to a mean half-life ($t_{½}$) of about 5 days.

The disparity between synthesis and loss rates derives from the fact that synthesis relates to the whole RNA pool whereas loss only reflects labelled RNA, which may be lost more quickly than the average rate if the RNA pool is heterogeneous.

When mRNA was removed from RNA fractions prior to analysis, similar, albeit slightly reduced, rates were obtained, about 6 and 12%/d for *s* and *l** respectively (Figure III.2.4C), confirming that mRNA only makes a small contribution to labelling at these time points. On basis of these results, we have shown that we are able to measure *in vivo* RNA turnover in CLL. More analyses of RNA turnover in CLL have been described in chapter 3 of the result section.

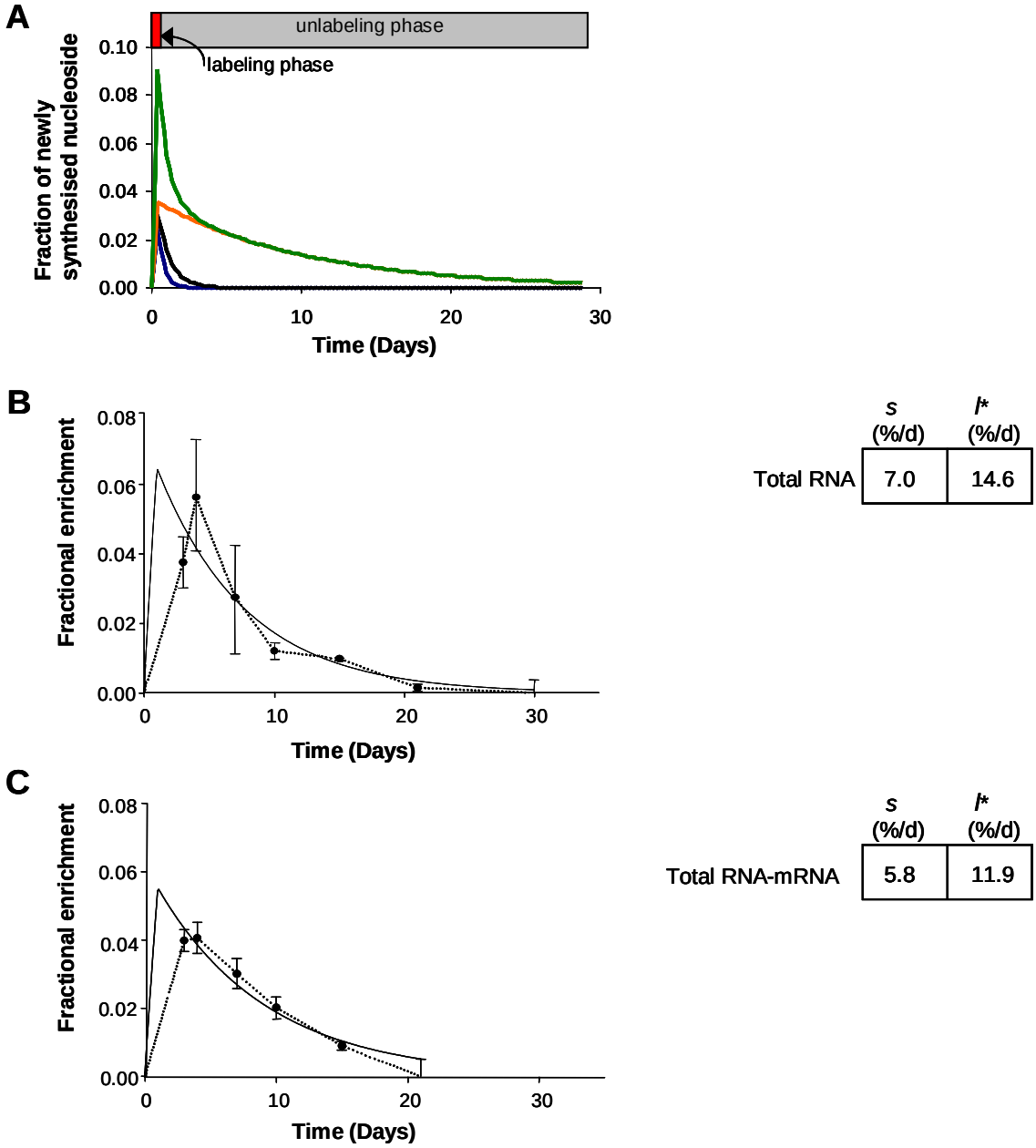

Figure III.2.4: In vivo labelling of RNA in CD3$^-$ lymphocytes from a subject with Chronic Lymphocytic Leukaemia

Theoretical model for contribution of mRNA (Blue), tRNA (Black), rRNA (Orange), to Total RNA (Green) labelling, the sum of the three components. Calculations assume that RNA comprises 4% mRNA, 8% tRNA and 88%rRNA, with half-lives of 0.3, 0.6, and 7 days respectively, and that each compartment behaves as a single homogenous compartment with equal synthesis and loss rate constants, k=s=l such that $F(t)=(1-e^{-kt})$ during labelling ($t\leq\tau$), and $F(t)=(1-e^{-k\tau}).e^{-k(t-\tau)}$ post-labelling ($t>\tau$), with a labelling period (τ) of 10 hours (A). Experimental data showing the fraction of labelled Total RNA (B), fraction of labelled [Total RNA-mRNA] (C) after oral labelling with [6,6-$^2H_2$]-glucose. Solid lines show curve fits generated using the model described. Inset data show modeled synthesis rates (*s*) for RNA and loss rates (*l**) for labelled RNA, as described.

2.4 Discussion

Turnover of RNA in cells is of interest for a number of reasons. In lymphocytes for example, it is of particular interest as it indicates metabolic activity, as opposed to proliferative activity. Thus terminally differentiated cells which lack the ability to divide may appear quiescent on proliferative assays but metabolic activity and RNA turnover are likely to be considerably accelerated. Investigation of the control of cell metabolic activities is important because changes in RNA content precede changes in protein metabolism (Grimble *et al*, 2000).

We describe here the development of a new stable (non-radioactive) isotope-mass spectrometric technique for the measurement of RNA synthesis and thus metabolic activity in dividing lymphocytes. As the technique involves no radioactivity or potentially toxic metabolites, it is suitable for use in humans as well as model systems. Previous approaches for the measurement of rRNA turnover *in vivo* using radioactive isotopes as ^{3}H-uridine are not widely applicable in clinical studies because of their toxicities. We have demonstrated that deuterium-labelled RNA through the *de novo* pathway may be analyzed in a similar way to DNA (Macallan *et al*, 1998). However we found that different derivatization conditions are required to generate the ATA derivatives of adenosine for GCMS analysis when compared to deoxyadenosine, this difference may be due to greater stability of the pentose ring of adenosine under derivatization conditions.

A similar approach, incorporating stable isotopic label through the *de novo* nucleoside synthesis pathway via glucose-6-phosphate, has been described to investigate intermediary metabolism of RNA (Boros *et al*, 2003). Boros et al used [1,2-$^{13}C_2$] - glucose labelling followed by analysis of the same derivative (aldonitrile triacetate), to investigate the effects of

transketolase activity in thiamine-responsive megaloblastic anemia (TRMA) on RNA precursor metabolism (Boros *et al*, 2003).

For turnover measurements, the [1,2-$^{13}C_2$]-labelled precursor may not be appropriate as the 1-carbon is lost during non-oxidative pentose production whereas the 6-position carbon of the ring is preserved making this the preferred site for labelling. In our studies *in vitro* using a lymphocyte cell line, we were able to demonstrate that adenosine is initially enriched much more quickly with deuterium than deoxyadenosine. This is probably due to rapid labelling of mRNA and possibly tRNA. The 3 species of RNA would be expected to label at different rates corresponding to their turnover; mRNA between 2-6 hours, tRNA around 24 hours and rRNA later. As the curves become parallel beyond 6 hours, we have deduced that most RNA production at this stage is related to new cell production, rather than production of new (ribosomal) RNA in existing cells. We were also able with this new approach to measure RNA turnover *in vivo* in one CLL patient and with a mathematical model to estimate synthesis rate of RNA (*s*) and rate of loss of labelled RNA (*l**) which predominantly reflects ribosomal RNA turnover. Synthesis rate of RNA of circulating B lymphocytes from one patient with chronic lymphocytic leukaemia appeared at faster rate of about 7%/d. Based on this only result in CLL, it is very difficult to drawn any conclusions as we didn't analyse more CLL patients and we didn't compare with healthy controls.

In terms of interpretation, several considerations apply, the most significant of which relates to the heterogeneity of RNA. Each RNA species will have its own rate of synthesis and degradation, and will contribute to the overall pool of labelled molecules in proportion to its abundance and its labelling rate. The measured value is therefore a composite as illustrated in Figure III.2.4A. Such a model is, of course, an over-simplification. RNA turnover is not a composite of three separate homogenous populations; considerable heterogeneity exists

within RNA moieties. Further method development should allow dissection of the relative contribution of different RNA species.

We separated mRNA from total RNA from CLL cells for analysis but did not obtain a sufficient signal for reliable quantitation of mRNA turnover, although we were able to measure the turnover rate of [Total–mRNA]. With more cells and/or more sensitive analysis, experiments with RNA species should be possible. In these studies we used samples of between 3 and 5 $x10^6$ cells; the minimum number of cells required for reliable analysis has yet to be established but will depend upon the RNA content of cells as well as their number. The time points used in the study of PBMC give some selectivity: samples taken greater than 3 days after labelling will predominantly reflect ribosomal RNA turnover, although interestingly there did still appear to be a small contribution from mRNA even 3 and 4 days post-labelling. Further experiments should include earlier time-points.

In terms of choice of label, for fast turnover moieties such as RNA, a precursor which is rapidly-incorporated and then rapidly disappears is required; glucose, with its short *in vivo* half-life, is an ideal candidate and may be given intravenously (Macallan *et al*, 2003;Macallan *et al*, 2005;Zhang *et al*, 2007) or orally (Ghattas *et al*, 2005;Defoiche *et al*, 2008). A universal issue in labelling studies is recirculation of label; this is likely to be minimal for deuterated glucose as glucose is rapidly metabolized and deuterium is primarily lost into the very large body water pool after oxidation. Although heavy water (^2H_2O) is useful for labelling DNA in slowly-dividing cells (Neese *et al*, 2002), where incorporation of deuterium takes place over weeks, it would not be suited to RNA analysis because of the relatively high rates of RNA turnover; RNA pools would become saturated with label and useful information would be obscured.

The main advantage of this technique over other approaches is its safety *in vivo* human studies; it is readily acceptable and without adverse effects or toxicities. Sufficient blood must be taken to allow separation of the cell of interest. The sensitivity of the method however, would allow other cell types to be analyzed in subjects with normal peripheral blood lymphocyte counts.

On basis of these results we conclude that a number of new applications for concurrent measurement of both metabolic and proliferative activity in the same cell populations are now possible using this approach.

3. Measurement *in vivo* of RNA turnover in chronic lymphocytic leukaemia

3.1 Introduction

CLL appears to be the consequence of imbalance of decreased cell turnover but what do we know on *in vivo* metabolic activity of these cells. We have shown that proliferation and death rate are reduced in CLL but does this correlate with a reduction on metabolic activity or are these cells simply non-dividing (but otherwise "active") or completely quiescent. We have developed a new approach for measuring RNA synthesis rates directly in human. If RNA turnover also indirectly reflects protein translation, this latter method thus allows evaluation of *in vivo* metabolic activity of CLL cells in patients.

Evidence on RNA metabolism in CLL cells is particularly limited. Using ^{14}C-uridine incorporation, Stilber et al found that ribosomal RNA synthesis in leucocytes from CLL patients is increased by 2 fold compared to normal patients (Silber *et al*, 1968). Experiments using tritiated uridine (^{3}HU) or methionine-methyl-tritium (^{3}HMe) suggested that the mechanisms regulating assembly of new ribosomes are defective in CLL cells (Rubin, 1971). To evaluate metabolic activity in B cells from CLL and healthy subjects, we therefore measured RNA turnover using the deuterium glucose labelling approach.

3.2 Materials and methods

3.2.1 Measurement and Analysis of deuterium enrichment

CLL samples used to measure RNA turnover were derived from our previous study on DNA turnover, diagnosis and characterization is summarized in appendix S1. The method of labelling and cell sorting is described in chapter 1. Total RNA from sorted $CD3^{-}CD19^{+}$ in CLL and healthy controls was extracted by guanidine thiocyanate / phenol extraction in a mono-phase solution (Trizol Sigma, St Louis, MO) and digested to yield adenosine which was converted to its aldonitrile triacetate derivative prior to analysis by GC-MS (monitoring ions m/z 256 and 258 by PCI in SIM; 6890/5973 GC-MS; Agilent Technologies, Bracknell, UK). Abundance-matched samples were analyzed in triplicate alongside a standard curve derivatized concurrently. Plasma glucose enrichment was analyzed using the same derivative (m/z 328 and m/z 330) to derive an area under curve for glucose-time which was taken as the precursor enrichment (*b,* in modeling below) except in one subject, CLL01, where plasma glucose enrichments were predicted from average glucose pool size and turnover rates from the other CLL subjects. Results were expressed as the fractional enrichment (F) and measured data was fitted to a model using nonlinear least squares regression (Sigmaplot v8.02; SPSS, Woking, United Kingdom) and solved for best fit values of *s* and *l** for RNA.

3.3 Results

3.3.1 Fraction of labelled RNA in B cell population

We compared RNA turnover in B lymphocytes subsets ($CD3^-$ $CD19^+$) in CLL and healthy subjects based on incorporation of deuterium into RNA of dividing cells. B cells were sorted by MACS and deuterium incorporation into RNA was quantified by GC/MS. As expected, in comparison with DNA, total RNA showed higher levels of labelling with a more rapid rate of loss consistent with faster turnover rate. When the time course of labelling was investigated, deuterium incorporation into RNA was found to peak between days 3 and 4 post-labelling and fall thereafter (figure III.3.1). Peak enrichment in CLL subjects, expressed as fractional enrichment (F) of labelled RNA, ranged from 3.0 to 5.4 %/day with a mean value of 4.29 %/day (table III.3.1). This was low compared to healthy controls, in whom fraction of labelled RNA per day was 7.11%/day (n=3). Despite the small numbers for comparison, this difference was statistically significant ($P<0.05$ by Wilcoxon-Mann-Whitney, two-tailed test).

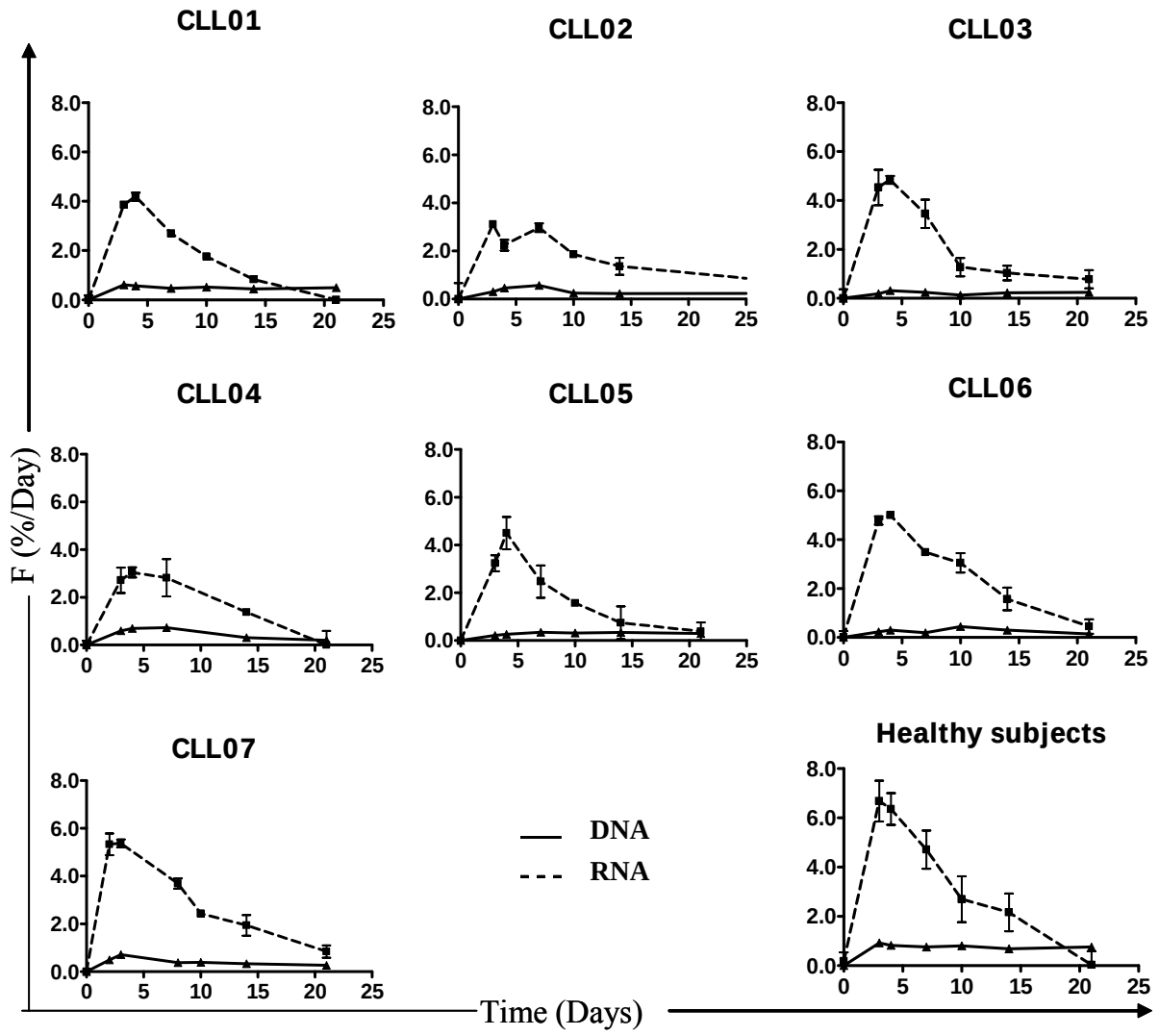

Figure III.3.1: Fraction of labelled Total RNA and DNA in CLL and healthy subjects.

Results show fraction of labelled total RNA and DNA in CLL and healthy subjects after labelling with [6,6-$^2H_2$]-glucose over a 10-h period. Note the error bars represent standard deviations of triplicate measurements by GC/MS for CLL subjects and the standard erros of the mean (SEM) for the healthy subjects.

Subjects	Ages	B cell number	Proliferation rate	Fraction of Labeled RNA	Synthesis rate of new RNA (*s*)	Doubling Time (T_2)	Disappearance Rate of labeled RNA (*l**)	Half life ($T_{1/2}$)
	Years	10^9 cells/l	%/day	%/day	%/day	day	%/day	day
CLL Patients (n=7)								
CLL01	44	144.5	0.58	4.19	7.71	9	15.90	4
CLL02	67	35.1	0.41	3.11	3.56	19	6.38	11
CLL03	69	99.7	0.24	4.84	6.78	10	12.74	5
CLL04	64	38.4	0.80	3.04	4.71	15	14.08	5
CLL05	65	89.8	0.29	4.50	5.65	12	12.50	6
CLL06	75	13.1	0.34	5.00	6.86	10	10.11	7
CLL07	49	42.8	0.63	5.37	6.43	11	8.39	8
CLLMean	62	66.2	0.47	4.29	5.96	12 [a]	11.44	6 [b]
SD		46.4	0.21	0.91	1.43	3.64	3.33	2.3
			*	*	*	*	NS	NS
Control Subjects (n=3)								
NMean	47	0.14	1.31 [c]	7.11	11.76	6 [a]	17.90	4 [b]
SD		0.12	0.83 [c]	1.59	1.04	0.55	9.96	1.9

Table III.3.1: Kinetics parameters of CLL and healthy subjects.

P<0.05 Wilcoxon-Mann-Whitney two tailed test between corresponding CLL and control parameters.
NS: P=0.67 according to Wilcoxon-Mann-Whitney two tailed test between corresponding CLL and control parameters.
[a] Mean doubling time T_2 = ln2/(mean *s*/100) was calculated from the mean value of synthesis rate *s*.
[b] Mean half-life time $T_{1/2}$ = ln2/(mean *l**/100) was calculated from the mean value of disappearance rate *l**.
[c] n = 7 (control subjects).

3.3.2 RNA turnover in CLL

Data from CLL and healthy subjects were modelled to derive synthesis and loss rate constants for labelled RNA in B lymphocyte subsets (*s* and *l** respectively) using the model described in chapter 2. As expected from the fraction of labelled RNA, total RNA synthesis rates in B-CLL cells were substantially lower than in healthy subjects (mean 5.96 %/d versus 11.76%/d; P< 0.05 Wilcoxon-Mann-Whitney, two tailed test, Table III.3.1, Figure III.3.2 and Figure III.3.3). Equivalent doubling times were 12 days and 6 days respectively. This suggests that metabolic activity, measured by RNA turnover, is reduced in CLL cells compared to B cells in control subjects.

When the rate of loss of labelled RNA (*l**) was calculated from modeling as described, loss rates were found to be substantially higher than synthesis rates (Figure III.3.3, Table III.3.1). This disparity is well recognized in DNA labelling studies (Macallan *et al*, 2005) and derives from the fact that the estimated synthesis rate relates to the whole RNA pool whereas the disappearance rate (*l**) relates only to loss of labelled RNA molecules. Rates of loss of labelled RNA (*l**) were lower in CLL subjects than in healthy subjects, 11.4 %/d, equivalent to a mean half-life ($t_{½}$) of about 6 days, compared to 17.9 %/day equivalent to half life of 4 days, in controls; this difference was not statistically significant (P=0.67, Wilcoxon-Mann-Whitney, two-tailed test, Table III.3.1).

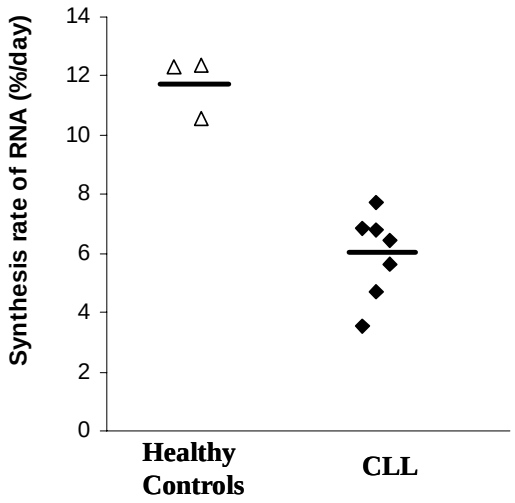

Figure III.3.2: Comparison of RNA turnover in CLL and in healthy subjects.

Results shown are synthesis rate of RNA (%/day) in CLL (◆) and in healthy controls (△) labelled with 6,6-$^{2}H_{2}$-glucose.

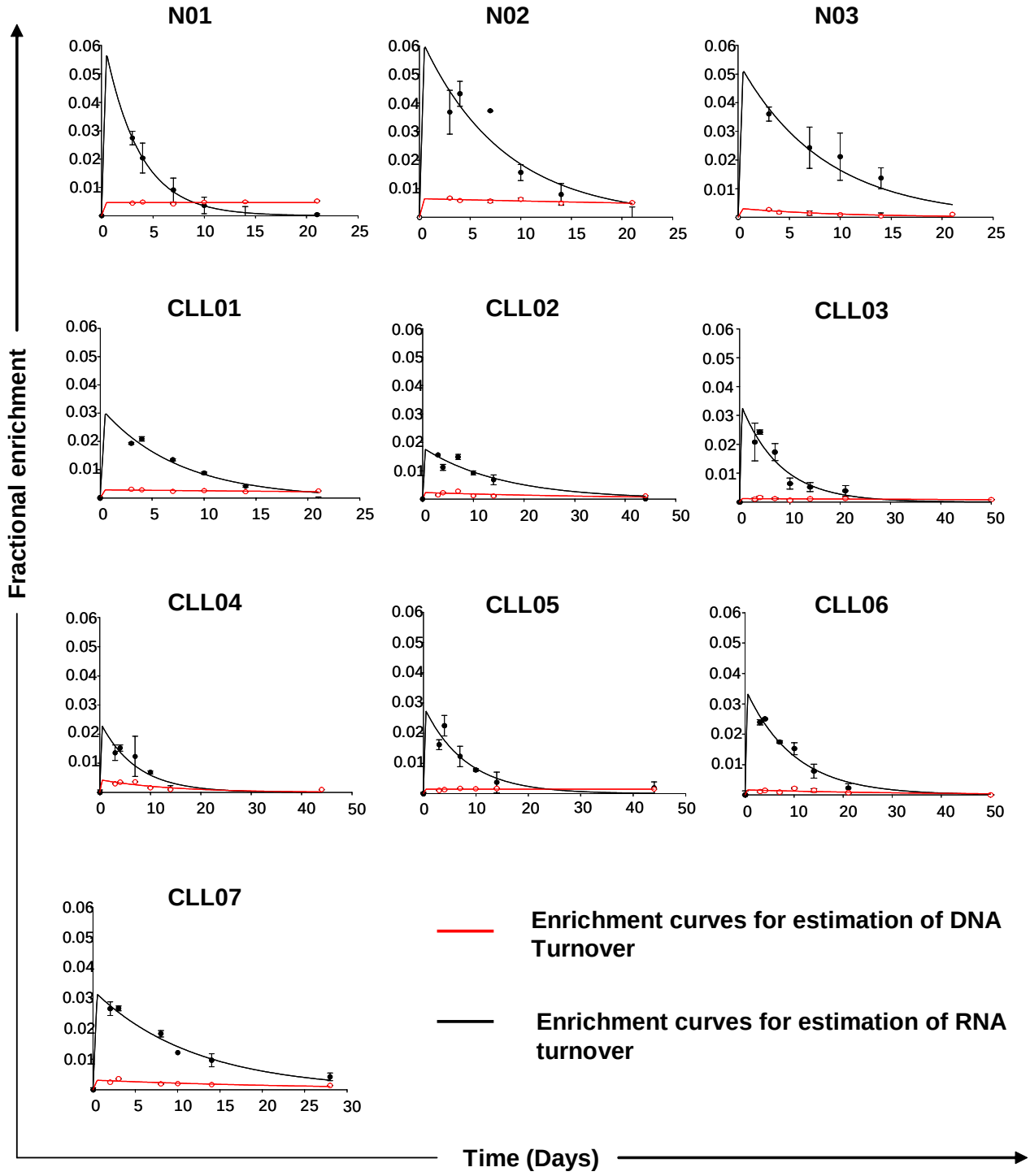

Figure III.3.3 Enrichment curves for DNA and RNA from healthy controls (n=3) and CLL subjects (n=7).

Values represent fraction of labelled deoxyadenosine and adenosine (F, as defined in mathematical *Modeling*) following oral administration of 6,6-$^{2}H_{2}$-glucose over a 10-h period. Error bars indicate standard deviations of triplicate measurements by GC/MS measurement of enrichment. Lines show best fit to model, assuming peak labelling at 12 hours.

3.4 Discussion

Several decades ago, early prospective studies using ^{3}HU, ^{3}HMe and ^{14}C-uridine indicated that RNA metabolism might be perturbed in CLL. The kinetics of ribosomal RNA transcription and processing were assessed in CLL cells incubated with PHA and labelled with methionine-methyl-tritium (^{3}HMe) and tritiated uridine (^{3}HU). It was concluded that mechanisms regulating assembly of new ribosomes in CLL were defective (Rubin, 1971). Other experiments using ^{14}C-uridine incorporation to measure Ribosomal RNA (28S, 18S, 5.8 S subunits) in leucocytes isolated from CLL patients have shown that RNA synthesis (28S and 5.8S) is increased two-fold compared to normal cells (Silber *et al*, 1968). In fact, their technique used ^{14}C-uridine incorporation to measure ribosomal RNA subunits in all leucocytes, not just CLL cells, and the short time period means that results are likely to include of time with the risk of labelling mRNA.

In our previous work, we have shown that proliferation and death rate are reduced in CLL but does those correlate with a reduction on metabolic activity. Since these studies have reported that RNA metabolism may be aberrant in culture *ex vivo* we measured RNA synthesis *in vivo* in order to understand whether RNA turnover is increased, reduced or unaltered in CLL patients. In this study, we compared RNA turnover in 7 CLL subjects and 3 healthy subjects using the deuterium glucose technique in order to determine if reduced B cell turnover is associated with a reduction in metabolic activity or if these cells are simply non-dividing but metabolically active. We have shown that synthesis rate (*s*) of RNA in CLL is significantly reduced compared to normal controls (5.96%/day versus 11.76%/day, $P<0.05$, Table III.3.1). Consequently the doubling time of RNA synthesis in CLL cells is extended by a factor of two-fold (12 days instead of 6 days in controls). Moreover, RNA was characterized by higher levels of labelling than DNA at all time point and the rate of loss of labelled RNA (l^*) was

lower in CLL, albeit not significantly different with these small numbers (11.44%/day versus 17.90%/day, P=0.67, Table III.3.1).

Circulating B lymphocytes from CLL subjects have shown a marked contrast between DNA and RNA kinetics. Such cells demonstrated very low rates of DNA turnover (0.35%/day), consistent with long survival (slow turnover) of cells as previously described. Based on the comparison of CLL cell turnover, we have suggested that RNA synthesis *in vivo* reflects cellular turnover of RNA rather than the synthesis of RNA related to cell division. Labelled RNA appeared at a much faster rate than DNA in such cells, with a synthesis rate of 5.96%/day. However, the "true" value may be higher as for RNA some early labelling may have been missed because blood was not sampled earlier than day 3 in line with previous studies of lymphocyte kinetics based on the lag between cell division and appearance in the circulation (Macallan *et al*, 2003). We have presumed that by the third day most labelled mRNA would have disappeared and the labelling would therefore comprise deuterium incorporated in both tRNA and rRNA, although probably predominately the latter.

The labelling protocol using deuterium glucose has shown that deuterium incorporation into RNA in CLL cells is higher than DNA and these cells make less RNA than controls. In contrast with other stable isotope requiring very long labelling as heavy water (^2H_2O), deuterium glucose is an ideal candidate for measuring fast turnover. One drawback of the deuterium incorporation technique used in this study is that sufficient amounts of cell populations are required to quantify turnover in RNA. In these studies we used samples of 3 $x10^6$ cells; the minimum number of cells required for reliable analysis has yet to be established and further exploration of alternative derivatives needs to be applied for CLL cells. Some preliminary work performed with a different derivative compound (PFTA derivative) was found to be more sensitive than the ATA derivative; starting amounts of adenosine for reliable measurement being about 0.5μg for PFTA and about 2μg for ATA. The

PFTA derivative would thus appear to be the optimal approach, especially as it would allow measurement of RNA species.

Further investigations on turnover rate of three RNA species but also more controls and important phenotypic should be still undertaken. Since proliferation rate is increased in $CD38^+$ cells, it will be interesting to measure metabolic activity of these cells but also seprated by ZAP-70 expression, another prognostic marker of the disease. Other phenotypic marker as CD27 will also need further investigation since in normal subjects CD27+ cells proliferate more rapidly than $CD27^-$. In our previous study, we showed that T cells kinetics is unaltered in CLL but due to a limited number of CLL patients we were unable to measure RNA turnover of T cells in CLL and healthy subjects.

Our report on measurement of metabolic activity in CLL indicates that RNA synthesis in CLL is reduced, as is the proliferation rate. We have been able to demonstrate that, not only are such cells relatively quiescent in proliferative terms with a lifespan of months, but they also have significantly reduced metabolic activity. In conclusion CLL appears to be the consequence of an imbalance of decreased cell turnover, resulting in increased dwell time in the circulation in association with metabolic quiescence signified by a significant reduction of RNA turnover. These *in vivo* studies indicate that CLL cells are quiescent with minimal proliferative and metabolic activity and these observations may impact on elaboration of efficient therapeutic strategies.

IV. Conclusions and prospects

Conceptually, the maintenance of stable B lymphocytes numbers results from a complex equilibrium between cell proliferation and cell death. A modification of one, or both, of these parameters might thus potentially lead to the accumulation of cells observed in CLL.

The first part of my thesis has been devoted to measure kinetics parameters involved in accumulation of B cells and to understand whether CLL is latent or proliferating disease. In this context, my experimental work aimed to compare B-cells kinetics in CLL subjects and in healthy controls based on incorporation of deuterium from 6,6-$^{2}H_{2}$-glucose into DNA. This study showed that daily proliferation rate of B cells in CLL is significantly reduced compared with healthy subjects but also that the disappearance rate tended to be lower in CLL than in controls. From these observations, we have extended two previous studies (Messmer *et al*, 2005;Macallan *et al*, 2005) that have suggested but did not prove due to different techniques that proliferation is reduced in CLL. Based on the measurement of cell turnover *in vivo*, these data indicate that the leukaemic B cell clone is continuously proliferating and dying at a reduced rate suggesting that chronic lymphocytic leukaemia is not a dynamic process as shown by Messmer et al.

In the second part of my thesis, we developed a novel approach for measuring RNA turnover *in vivo* using using the same labelled samples previously used for DNA analysis. We have determined that derivatization conditions for the analysis of deuterium incorporation into RNA with GC/MS were different from those required for DNA, due to greater stability of the pentose ring of adenosine under derivatization conditions. Our experiments *in vitro* with PM1 cells have shown that deuterium incorporation into RNA is higher than in DNA and that RNA production is related to new cell production. Moreover we have constructed a model estimating the relative contributions of mRNA, tRNA, and rRNA to the total labelling curve. We have deduced from our *in vivo* experiments performed in one CLL patient that PBMC labelling

curves more than 3 days post-labelling reflect almost exclusively changes in ribosomal RNA labelling.

In the last part of my thesis, we applied this new approach in a pilot study to measure RNA synthesis in CLL and in healthy individuals. CLL cells *in vivo* have slower proliferation rates suggesting that RNA synthesis *in vivo* reflects cellular turnover of RNA rather than the production of new RNA related to cell division. We found that RNA is enriched much more quickly with deuterium than DNA at all time points. The comparison between CLL and healthy subjects revealed that RNA synthesis is significantly reduced in CLL compared to normal controls. Based on these observations, CLL appears to be the consequence of an imbalance of decreased cell turnover and metabolic activity. In conclusion, our work has cast the light onto cell turnover and RNA turnover in CLL. These *in vivo* studies indicate that CLL cells are quiescent with minimal proliferative and metabolic activity.

This thesis opens potential prospects for the development of a new prognostic marker. In fact, most CLL patients require no treatment because of an indolent course while other patients become symptomatic or develop signs of rapid progression. Chronic lymphocytic leukaemia is still an incurable disease and the choice of the first-line treatment depends on the patient's risk factor. Younger patients or those characterized by poor prognostic factors may benefit of an early and severe treatment. A major problem in managing of CLL is to decide between the "wait and watch" or early treatment options. There are presently no available prognostic markers that appropriately address this key question. Since DNA and RNA turnover rates are important parameters in CLL biology, we propose to assess their prognostic value. In this context, a correlation between proliferation rates and disease progression is currently being evaluated as an interesting potential prognostic biomarker for CLL; ongoing study NIH (Murphy e*t al*, 2007).

Data described in this thesis show that CLL cells turnover less frequently and have reduced metabolic activity. Therefore, we propose that an efficient therapeutic approach would restore normal DNA and RNA synthesis rates. One possible strategy would include gene activation with HDAC inhibitors. Indeed, previous works have reported that VPA treatment was efficient for leukaemia/lymphoma therapy in the sheep model leading to decrease lymphocyte numbers and tumor regression (Achachi *et al*, 2005). In addition VPA induces apoptosis of B-CLL cells through caspase activation at concentration achievable in humans (Baya Bouzar *et al*, 2008). Moreover concomitant incubation of B-CLL cells in the presence of fludarabine or cladribine with VPA induces a significant increase of apoptosis compared to PNAs alone. Therefore, VPA could improve leukaemic cell clearance and restore response in refractory patients via concomitant stimulation of complementary apoptotic pathways. A clinical phase II trial using VPA and cladribine is initiated to evaluate this promosing therapy.

V. References

5. Publications

5.1 Article in preparation :

Defoiche J., Zhang Y., Lagneaux L., Pettengell R., Willems L., Macallan D. (2009). Reduction of ribosomal RNA turnover *in vivo* in patients with chronic lymphocytic leukemia. In preparation

5.2 Submitted papers:

Defoiche J., Bouzar B.A., Macallan D. & Willems L. (2009). Dameshek's dogma: not dead yet. *Submitted to British Journal of Haematology*.

Defoiche J., Zhang Y., Lagneaux L., Pettengell R., Willems L., Macallan D. (2008). Measurement of ribosomal RNA turnover *in vivo* using deuterium-labelled glucose. *Submitted to Clinical Chemistry.*

Macallan D., Asquith B., Zhang Z., de Lara C., Ghattas H., **Defoiche J**., Beverley P. (2008). Measurement of proliferation and disappearance of rapidly turnover cell populations using deuterium-labelled glucose. *Submitted to Nature Protocols.*

5.3 Accepted papers:

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