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# Analysis of intratumoral immune parameters associated with the micrometastatic state in patients with colorectal cancer

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Title (English)

## Analysis of intratumoral immune parameters associated with the micrometastatic state in patients with colorectal cancer

Title (Swedish)


Abstract

The immune infiltrate present in colon cancers was estimated in a large series of tumour specimens in correlation with the presence of micrometastases. Tissue specimens were homogenised and RNA was extracted with the RNeasy® technology. The RNA was then reversely transcribed to cDNA and the expression determined with TaqMan™ real time quantitative PCR (low density arrays). The preliminary results indicated an association between the amount of CD8 expressing cells and the defence orientation ($T_H1$) with the micrometastatic state.



Keywords

Colon cancer, micrometastases, immune infiltrate, lymphocyte, quantitative PCR



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# Analysis of intratumoral immune parameters associated with the micrometastatic state in patients with colorectal cancer

**Malin Nilsson**

## Sammanfattning

Tjocktarmscancer är en av de mest förekommande cancertyperna. Ofta sprids cancern till andra delar av kroppen. För att detta ska ske krävs att celler lämnar modertumören, antingen en och en eller i kluster. Dessa första celler kan observeras i blodkärl, lymfkärl eller längs nervstrukturer nära modertumören och kallas mikrometastaser. Förändrade celler ska egentligen oskadliggöras av kroppens immunförsvar men tumörceller undkommer på olika sätt detta försvar. I denna studie har ett antal immunologiska faktorers närvaro undersökts i en serie prov från tjocktarmstumörer. Detta resultat jämfördes med närvaron eller frånvaron av mikrometastaser hos dessa patienter. De preliminära resultaten tyder på att mängden cytotoxiska T-celler och en inriktning av T-hjälparcellerna mot $T_H1$-försvar kan vara korrelerat till det mikrometastasiska tillståndet.

**Examensarbete 20p i Molekylär Bioteknikprogrammet**
**Uppsala universitet februari 2005**

## TABLE OF CONTENTS


**1. Introduction ..... 4**
- **1.1 Epidemiology of colon cancer ..... 4**
- **1.2 Colon cancer – the disease ..... 4**
- **1.3 The micrometastatic process in colon cancer ..... 4**
- **1.4 The Immune System – an overview ..... 5**
- **1.5 Immune response and cancer ..... 6**
- **1.6 Immune response and colon cancer ..... 7**
- **1.7 The scientific question ..... 8**
- **1.8 Aim of the study ..... 8**
- **1.9 Previous work ..... 8**
  - **1.9.1 Immune infiltrate in colon cancer ..... 8**
  - **1.9.2 Analysis of the immune cells in colon cancer in relation with micrometastasis ..... 9**

**2. Materials and methods ..... 10**
- **2.1 Tumour samples processing ..... 10**
- **2.2 Patients ..... 10**
- **2.3 Homogenization ..... 10**
- **2.4 RNA extractions ..... 11**
- **2.5 Analysis of the RNA quality ..... 11**
- **2.6 cDNA ..... 12**
- **2.7 Real-time quantitative RT-PCR ..... 12**

**3. Results ..... 14**
- **3.1 Comparative analysis of two RNA extraction kits ..... 14**
- **3.2 The cDNAs and genes included in the study ..... 17**
  - **3.2.1 The selected cDNAs ..... 17**
  - **3.2.2 The selected genes ..... 17**
- **3.3 Quality control of the amplifications ..... 17**
  - **3.3.1 Reproducibility ..... 17**
  - **3.3.2 Homogeneity of the curves ..... 18**
- **3.5 Control of immunologic profiles observed with the low density arrays .. 20**
- **3.6 Calculations with the obtained data ..... 22**
  - **3.6.1 Amplification curves ..... 22**
  - **3.6.2 Preliminary results ..... 23**

**4. Discussion ..... 30**

**5. References ..... 34**

**6. Appendices ..... 36**
- **6.1 Appendix I ..... 36**
- **6.2 Appendix II ..... 37**
- **6.3 Appendix III ..... 38**

**7. Acknowledgements ..... 39**

# INTRODUCTION

## 1.1 Epidemiology of colon cancer

Cancers of the colon are the fourth most common type of cancer and affects annually about 800 000 individuals over the world. The populations of Europe, North America and Australia are more affected possibly due to their life style; a diet poor in fibres and rich in saturated fats accompanied by little or no exercise.[1] The majority of the colon cancer cases are sporadic cases, approximately 10% are inherited. The average age of diagnosis is quite high, between 60 and 70 years.[2]

## 1.2 Colon cancer – the disease

Colon cancer is a mucosal disease, which means that all colon cancers are derived from the mucosal lining of the bowel wall. The bowel wall is composed of several layers: the mucosa, the submucosa, the muscularis propria and the serosa. The earliest genetic changes that lead to the development of cancer cells take place in the mucosa, which is a single layer of columnar epithelial cells. This is the site where cells are continuously dividing, a process that begins in the crypts of Lieberkuhn. Under this layer lies the submucosa that contains blood- and lymph vessels as well as terminal nerve fibers, and is therefore a layer of importance in the development of tumours. Once the tumour has invaded this layer the risk of distant spreading increases.

The transformation of a normal cell to a cancerous one is a process of multiple steps and involves changes in the expression of several cancer associated genes; the proto-oncogenes and the tumour suppressor genes. Proto-oncogenes induce cellular proliferation and are involved in the regulation of apoptosis. Tumour suppressor genes are inhibitors of cellular proliferation. Colon cancer progresses in a series of well-defined morphologic stages. The colon cancer begins as small benign tumours, on the lining of the colon, called adenomas. These precancerous tumours grow, successively becoming more and more disorganised in their intracellular organisation until they acquire the malignant phenotype. The well-defined morphologic stages of colon cancer have been correlated with a sequence of genetic changes involving inactivation or loss of three tumour suppressor genes (*APC, DCC, p53*) and activation of one cellular proliferation oncogene (*K-ras*).[3, 4]

## 1.3 The micrometastatic process in colon cancer

Cancer cells disseminate from the primary tumour either as individual cells or in clusters. The migration is typically regulated by integrins (a large family of cell adhesion receptors), matrix degrading enzymes, cell-cell adhesion molecules and cell-cell communication. Extra-cellular matrix degrading enzymes such as matrix metalloproteinases (MMPs) are frequently up-regulated in tumour cells and facilitate migration *in vitro* and metastasis *in vivo*. In colon carcinoma the collective migration is commonly used and exists in two forms; the first variant is when formations of multiple cells protrude yet stay in contact with the primary tumour, a so called local invasion. The second variant is detached cell clusters (or micrometastases) that extend along interstitial tissue gaps and paths of least resistance as well as along perineural structures, lymphatic vessels and blood vessels. Due to changes in the microenvironment, such as changes in the expression of proteases and integrins or due to drugs such as protease- or MMPs inhibitors, cells can develop migratory escape strategies. In epithelial cancers of colon, following tumour progression and dedifferentiation, the cancer cells can undergo a transition from a collective invasion pattern towards a detached and disseminated cell migration mechanism. The cells lose their cell to cell junctions but retain expression of migration promoting molecules. This transition is thought to be a significant step in the

invasive cascade, once the cancer cells spread as single cells the metastatic spread is increased, resulting in poor prognosis.[5]

### 1.4 The Immune System – an overview

Immunity is divided into two components, innate and adaptive immunity. These two are not independent of each other; they work together in a coordinated way to eliminate invaders. The innate immune system provides the first line of defence and can be seen to include four types of defensive barriers: the anatomic, the physiologic, the phagocytic and the inflammatory. Most of the components of innate immunity are always present and are not specific to any pathogen but rather to groups of pathogens. Important actors in the innate defence are for example the skin, mucous membranes, saliva, tears, mucous secretions, pH, specific molecules (lysozyme, interferon and complement), phagocytosis by specialized cells (monocytes, macrophages, neutrophils, eosinophils) or inflammatory responses.

The adaptive response is, unlike the innate immune defence, a reaction to a specific antigenic challenge; subtle differences among antigens can be distinguished. The adaptive response is also different from the innate in that it shows a vast diversity in the production of recognition molecules, it has memory and is able to discriminate between self and non-self. The attributes of the adaptive immune system are a consequence of the lymphocytes' production and expression of antigen-binding cell surface receptors. For the adaptive response to function cooperation between these lymphocytes and antigen-presenting cells is essential. B lymphocytes and T lymphocytes are the two major populations of lymphocytes.

The mature B lymphocyte expresses a unique antigen-binding receptor on its membrane; a membrane-bound antibody molecule. Upon antigen recognition of a naïve B cell the cell divides rapidly, its progeny differentiates into memory B cells and effector B cells (plasma cells). The effector cells express few membrane-bound antibodies but produce large amounts of antibodies in secreted form and these plasma cells are the major effector molecule of humoral immunity.

Every T lymphocyte has a unique antigen-binding molecule called the T cell receptor (TCR). The TCR can only recognise antigens bound to cell membrane proteins called major histocompability complex (MHC) molecules. The MHC is referred to as the HLA complex in humans. There are two classes of MHC molecules and they are found on cell membranes. MHC class I molecules are expressed by nearly all nucleated cells and MHC class II are mostly expressed by antigen-presenting cells (APC), such as macrophages, B lymphocytes and dendritic cells. There are two well-defined subpopulations of T cells: T helper cells ($T_H$) and T cytotoxic cells ($T_C$). These subpopulations can be distinguished from one another by the presence of either CD4 or CD8 membrane glycoproteins on their surfaces: CD4 on $T_H$ cells and CD8 on $T_C$ cells. $T_H$ cells recognise antigen bound to MHC class II on the surface of APCs and $T_C$ cells recognize antigen bound to MHC class I. CD4 is a transmembrane monomeric glycoprotein. CD8 occurs usually as a heterodimer of two subunits called CD8-α and CD8-β but can transitorily be expressed as a disulfide-linked homodimer of CD8-α subunits.

The chain of events in the adaptive response begins with an activated T helper cell. For a naïve T helper cell to become activated a costimulatory signal is necessary in addition to the initial signal that arises upon antigen recognition by the TCR. This signal is provided primarily by interactions between a protein, CD28, on the $T_H$ cell and a molecule of the B7 family on the antigen-presenting cell. The activated $T_H$ cell is an effector cell that secretes

various growth factors known collectively as cytokines. The secreted cytokines play an important role in activating B cells, $T_C$ cells, macrophages and other cells that participate in the immune response. Different patterns of produced cytokines by T helper cells result in different types of response. CD4 effector T cells form two subpopulations, the $T_H1$ and $T_H2$ T cell subsets, which are separated by the different panels of cytokines they secrete. $T_H1$ preferentially produces IL-2, IFN-γ and TNF-β resulting in a cell-mediated response. $T_H2$ cells produce IL-4, IL-5, IL-6 and IL-10 and stimulate an antibody response. These subsets also regulate each other; IFN-γ secreted by $T_H1$ can inhibit the responsiveness by $T_H2$ cells, and in the same way IL-10 produced by $T_H2$ down-regulates B7 and IL-12 expression by APCs, which in turn inhibits $T_H1$ activation. IFN-γ also influences the subset development, in favour for $T_H1$. Hence differentiation into $T_H1$ and $T_H2$ is regulated by cytokines but transcription factors also have an important role. $T_H1$ differentiation is controlled by transcription factors such as T-bet, Stat1, and Stat4. $T_H2$ differentiation is promoted by IL-4 and the transcription factors Stat6, GATA-3 and c-Maf.[6] It has been reported that T-bet induces expression of IFN-γ. T-bet also activates other $T_H1$ genetic programs and at the same time it represses the opposing $T_H2$ programs.[7]

Under the influence of $T_H1$ derived cytokines a $T_C$ cell that recognizes an antigen proliferates and differentiates into an effector cell called a cytotoxic T lymphocyte (CTL). The CTL does not secrete many cytokines; instead it exhibits cell killing and cytotoxic activity. The CTLs monitor the MHC class I positive cells of the body and can eliminate cells that display foreign antigen such as virus-infected cells or cells of an unknown tissue graft. CTLs also have the capacity to eliminate tumour cells that express altered self-antigens.

The third subgroup of lymphocytes is the natural killer (NK) cells. NK cells recognise its targets in different ways. By membrane receptors the NK cell can distinguish abnormalities such as a reduction of the display of MHC I or unusual profiles of surface antigens displayed by some cancer or virus infected cells. NK cells can also recognise antibody-coated cells. The NK cells can attach to these antiviral or antitumoral antibodies and subsequently destroy the target cell. This is called antibody-dependent cell-mediated cytotoxicity (ADCC).

### 1.5 Immune response and cancer

An immune infiltrate is present in and around tumours. In the debate regarding whether the immune system is implicated or not in the fight of cancer several arguments have been presented supporting its protective role. The theory of immune surveillance states that tumours arise only if cancer cells are able to escape immune defence either by reducing expression of tumour antigens or by impairment in the immune response to these cells. Several arguments are supporting this theory. For instance, immunodeficient mice have been shown to suffer more frequently from spontaneously occurring cancers[8] and human cancers are more frequent in patients with a weak immune defence, for example in patients taking immunosuppressive drugs or in elderly persons. Immunotherapy in clinical assays with IL-2, tumour infiltrating lymphocytes and vaccinations has concluded, although modestly, that immune factors have an impact[9]. Hence, potentially, immune effectors are capable of attacking and destroying a tumour. It has also been shown that cytotoxic cells have an important role in this defence and several antigens potentially recognised by CD8 T cells have recently been identified.[12] These antigens can be of several types; antigens encoded by genes exclusively expressed by tumours, antigens encoded by normal genes that have been altered by a mutation, antigens normally expressed only at a certain stage of differentiation or by certain differentiation lineages and antigens that are overexpressed in particular tumours. The immune surveillance theory has still not been demonstrated in humans, clear is anyhow that

an immune response can be generated to tumour cells. A number of tumours have been shown to induce tumour-specific cytotoxic T lymphocytes that recognise tumour antigens presented by class I MHC. The cell-mediated response appears to play a major role but due to reduced MHC expression, as reported in many cancers, the role of specific CTLs in their destruction is limited. Since recognition of tumour cells by NK cells and macrophages is not MHC restricted it is not compromised by decreased MHC expression. Tumour infiltrating macrophages can contribute to tumour rejection but can also be essential for tumour growth or occur as innocent bystander cells in tumours[10]. The anti-tumour activity of activated macrophages is presumably mediated by lytic enzymes and reactive oxygen and nitrogen intermediates. In addition activated macrophages secrete a cytokine called tumour necrosis factor (TNF-α) that has potent antitumour activity. The macrophages may however also exhibit tumour promoting activity and have a role in for example angiogenesis[11]. Tumours show several mechanisms to escape immune responses. As already mentioned the partial or complete loss of expression of class I MHC molecules is one way the tumour can escape T cell control. Tumour cells may lack co-stimulatory receptors, and thereby cause the T cells to become anergic. Tumour cells may also lack other molecules, necessary for adhesion of lymphocytes such as for example ICAM-1 or express anti-adhesive molecules. Moreover it has been observed that tumour-specific antigens temporarily can disappear from the surface of the tumour cell when the appropriate antibody is present. Yet another way for cancer cell to evade rejection is by making immunosuppressive cytokines as TGF-β or IL-10. TGF-β suppresses inflammatory T cell responses and cell mediated immunity and IL-10 can reduce dendritic cell development and activity.

### 1.6 Immune response and colon cancer

Colon cancer was for long considered as poorly immunogenic, however solid colon tumours may express tumour associated antigens (TAA) that T cells can recognize and immune responses against tumours in colon cancer patients might take place. These anti-tumour immune responses can have an influence on patient prognosis.[12]

The protective role of inflammatory infiltrates has not yet been clearly demonstrated. Concerning the number of eosinophils, mast cells and γδ T cells present in colon tumours their common feature seem to be a decrease in quantity as the tumour progresses. Their presence might indicate a less advanced tumour and not an actual anti-tumour response. Regarding the role of macrophages, their number in colon tumours is higher than in normal colorectal mucosa. This might however be the result of an active recruitment by the tumour and not a directed immune response. Tumours make use of their pro-angiogenic and prometastatic effects; macrophages can produce angiogenic and immunosuppressive growth factors such as TGF-ß and can promote tissue remodelling by secreting matrix metalloproteinases. Macrophages at the tumour site may therefore promote colon tumour progression and metastasis. The number of dendritic cells (antigen presenting cell and primary T cell activator) is yet another example of an important immune cell whose presence decreases in colon tumours.

The presence of lymphoid infiltrates indicates however a sign of systematic anti-tumour immune response that might have a positive prognostic role. The subsets of most interest are the T cells since the colorectal mucosa normally is very rich in B cells. Their phenotype (active or naïve) as well as the specific localisation of the infiltrate might also be important to evaluate their prognostic value. The presence of intra-epithelial cytotoxic T cells has been shown to be a strong predictor of better survival. It has been reported that intra-epithelial CD8 T cells in colorectal carcinoma display a higher expression of molecules involved in target

cell killing such as perforin and granzyme B and have a higher proliferative activity than those present in normal mucosa proposing that they are active effector cells (CTLs).[12] In the matter of $T_H$ cells their characteristics in colon cancers is not fully described. The $T_H$ cells are of a major importance for the carefully directed events that result in a protective immune response. These events are to a large extent coordinated by cytokines produced by $T_H1$ and $T_H2$ T-cell subsets as described earlier. Colon cancer patients often show low levels of the $T_H1$ type immunity.

### 1.7 The scientific question

The microinvasive process is an event that precedes distant spread and metastases. Does the nature and quality of the immune defense intervene in the control of these events? In our knowledge this question is not or at least incompletely answered.

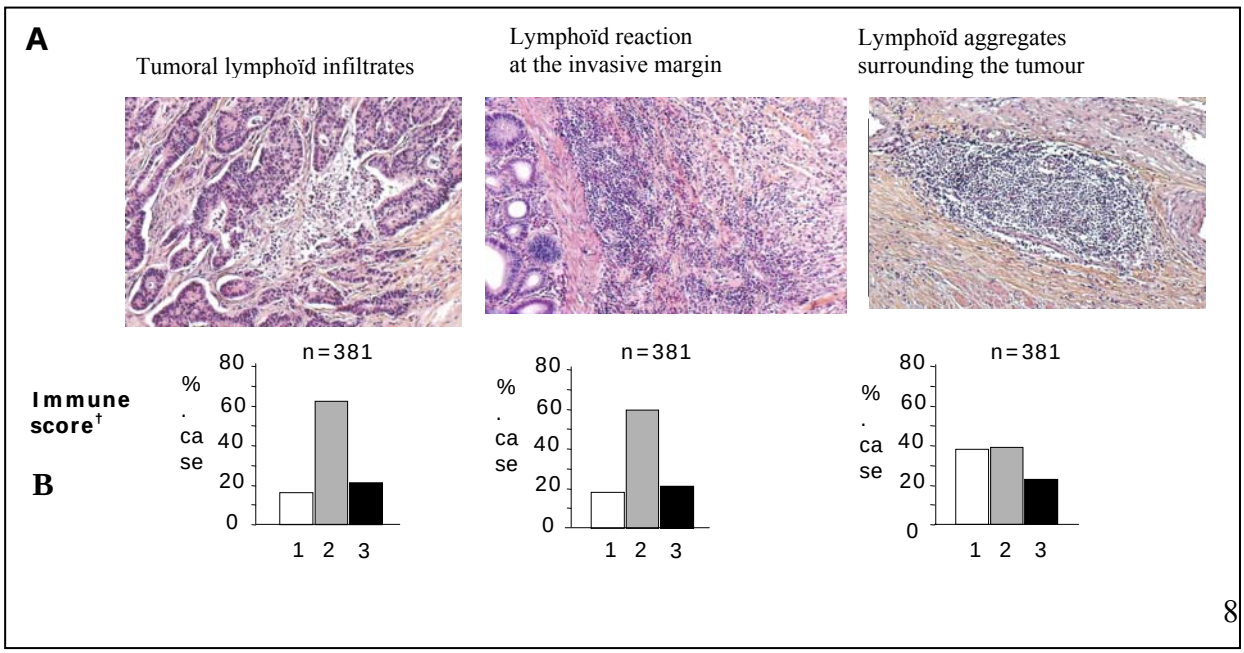
### 1.8 Aim of the study

The objective with this work was to analyse the immune infiltrate present in colon cancers in a large series of tumour specimens in correlation with the presence of micrometastases. Prior to this study a collection of tumours had been analysed with flow cytometry and immunohistological chemistry. Within the degree project the technique used to investigate the immune infiltrate present was real time quantitative PCR. The obtained results will later on be analysed contingent on data obtained from flow cytometry experiences for 40 patients and validated by the immunohistological technique using a Tissue Microarray (TMA).

### 1.9 Previous work

#### 1.9.1 Immune infiltrate in colon cancer

The team that I worked with during my degree project had already looked closer into the state of the immune infiltrate in colon cancer. Following surgical removal of colon cancer, tumoral tissue is routinely embedded in paraffin by the Anatomopathologic service at the HEGP (Hopital Europeen Georges Pompidou). 5µm sections of these paraffin blocks are then stained with HE (hematoxylin eosine) for histologic diagnosis. The immunologic infiltrate for a series of 381 patients (these patients corresponded to three years of surgical work and therefore included all stages of cancers) was analysed by re-inspecting such sections. The variability of the immune infiltrate was examined in three distinct zones: the centre of the tumour, the invasive margin of the tumour and at distance from the tumour in lymphoid aggregates (figure 1.1A). The intensity of the infiltrate was classified into a three-grade scale: 1 for a weak immune infiltrate, 2 for a medium immune infiltrate and 3 for a strong immune infiltrate (figure 1.1B).



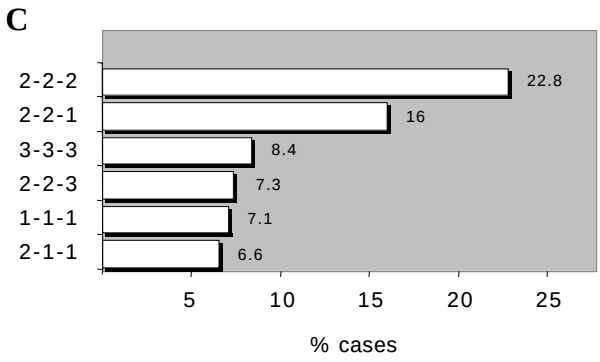



**Figure 1.1**
**A** The three zones analysed; the centre of the tumour, the invasive margin of the tumour and a section at distance from the tumour in lymphoid aggregates. The pictures show HE stained sections.
**B** The percentage of patients exhibiting the immune scores 1(weak immune infiltrate), 2 (medium infiltrate) and 3 (strong infiltrate) in the different zones.
**C** The 6 most frequent combinations of scores and the percentage of patients demonstrating these scores is shown. The three letter code describes the infiltrate in the order: the centre of the tumour, the invasive margin of the tumour and at distance from the tumour in lymphoid aggregates.

From these results it was observed that the immune infiltrate was variable in the cancer specimens from the patients of the series. It was also observed that 55% of the tumours had a non-homogenous immune infiltrate in the three different zones analysed (data not shown). The 6 most common combinations of scores in the three zones are shown in figure 1.1C in the order: the centre of the tumour, the invasive margin of the tumour and at distance from the tumour in lymphoid aggregates.

### 1.9.2 Analysis of the immune cells in colon cancer in relation with micrometastasis

The immune populations of 40 fresh tumour specimens had been analysed with flow cytometry (figure 1.2). Whole cell populations extracted from the tumours were stained with antibodies against CD3, TCRαβ, CD4, CD8, CD56, CD19, CD14 and CD1a. The percentage of each population of positive cells among the living cells of the extract was calculated. The correlation between the percentage of these populations and the histopathological findings were analysed. The assembly of immunitary populations studied appeared to be more present in cancers without micrometastases.

The results showed that the percentage of cells expressing CD3, TCRαβ, CD4 and CD8 within the tumour was significantly correlated with the micrometastatic state (i.e. the presence of micrometastases in perineural structures, lymphatic vessels and blood vessels). (p values < 0.05).

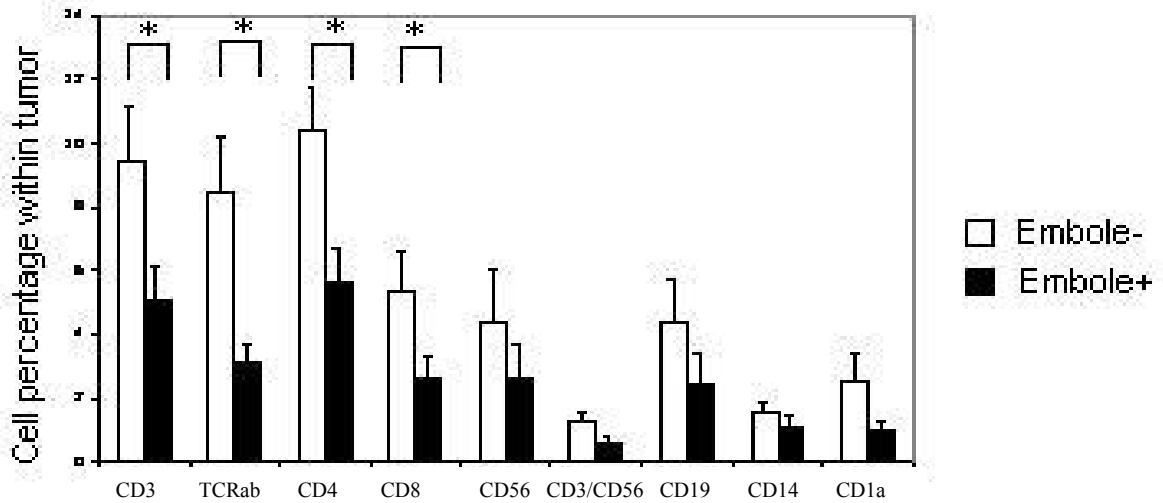



**Figure 1.2** Analysis with flow cytometry. The white columns are patients without micrometaastases and the black columns those with micrometastases present (Embôle is french for micrometastases) . The diagram shows the results from staining with antibodies for (from the left) CD3, TCRab, CD4, CD8, CD56, CD3/CD56, CD19, CD14 and CD1a. A high percentage of CD3, TCR ab, CD4 and CD8 expressing cells was significantly associated with the absence of micrometastases. * represents a p value <0,05 (statistic test: Student-t).

In summary, the results of the previous work presented here demonstrated that the immune infiltrate was heterogeneous in aspect and density in colon cancers and that the nature of the immune populations present at the tumour site could influence the microinvasive process.

# MATERIALS AND METHODS

## 2.1 Tumour samples processing

The tumours included in this project were obtained from HEGP (Hopital Europeen Georges Pompidou) in Paris. Directly after surgery small pieces of the tumours were cut and frozen in liquid nitrogen. The tissues from the patients were then made anonymous by the creation of a code consisting of the first three letters of the given family name and the first three letters of the name. The achieved frozen tissue specimens were then divided in three pieces to obtain 20-30mg for the RNA extraction, a slightly bigger fragments for a protein extraction (40-50mg) and one small fragment from each patient was to put in cryomatrix (frozen specimen embedding medium from ThermoShandon) for a histologic control. All handling of the tumours was performed in the vapour of liquid nitrogen to avoid thawing. The fragments for the extractions were put into 2-ml sterile round-bottomed skirtless tubes (Treff Lab, Degersheim, Switzerland) with sterile microbeads (5mm in diameter, stainless steel, Qiagen, Courtaboeuf, France) and put in a freezer (-80°C) prior to homogenization of the tissues.

## 2.2 Patients

The patients included in this study were classified according to specified criteria. The classification had been done following the routinely performed histopathologic examination of the tumour. During this examination the stage of the tumour i.e. the depth and invasiveness was determined, as well as the number of invaded lymph nodes and the presence or absence of metastases. The presence or absence of tumour cells in lymphatic vessels, blood vessels or along perineural structures was also observed. The classification (TNM and Duke's) along with the histopathologic characteristics of the series are included in Appendices II-III.

## 2.3 Homogenization

The homogenization (disruption) was performed with a Mixer Mill (Qiagen). It comprised two racks (in total 48 vessels could be proceeded simultaneously). Round-bottomed skirtless sterile Eppendorf tubes containing beads and tissue were put in the racks. The racks were then shaken with a certain frequency. The disruption was made in series of at most 24 samples at a time, i.e. corresponding to one complete rack. The frozen tissue was disrupted without additions in a rack pre-cooled in liquid nitrogen before adding of the lysis solution. The duration of the disruption was a compromise between avoiding thawing and getting the finest powder possible.

In the final procedure one rack was put in liquid nitrogen while placing tubes in it. Thereafter the rack was put into the Mixer Mill for 45s at 30Hz, equilibrated with a second rack. This step was repeated after a quick recooling of the rack in liquid nitrogen. Then 1ml of lysis solution[i] was added and the tubes were put in another rack at RT (this time to avoid the freezing of the lysis solution and the tissue) and then shaken once more at 30Hz for 30s enabling an efficient mixture of the powdered tissue and the solution. Hence, the final protocol included 2 x 45s at 30Hz and 1 x 30s at 30Hz after the addition of the lysis buffer.

[i] Nucleic Acid Purification lysis solution (Applied Biosystems), supplied as 2X solution and diluted with 1 volume of calcium/magnesuim-free phospate buffered saline (PBS) before use *or* Buffer RLT (Qiagen) with 10µl β-Mercaptoethanol / ml Buffer RLT.

The lysis buffer from Applied Biosystems contained a detergent that caused foaming and the samples were therefore put on ice for 30 minutes to allow the foam to subside. The lysate was split into two tubes and frozen in -80°C prior to RNA-extraction.

### 2.4 RNA extractions

*ABI PRISM™ 6100 Nucleic Acid Prep Station*

The RNA extractions were performed with the ABI PRISM™ 6100 Nucleic Acid Prep Station (Applied Biosystems, Courtaboeuf, France). Samples and wash reagents were added manually. The tissue lysates were transferred to trays and the samples passed through under controlled vacuum conditions. To reduce the risk of clogging wells in the purification tray (Applied Biosystems), the samples were treated with Proteinase K (200µg/ml lysis buffer) (Applied Biosystems) for one hour in RT and prefiltered before the isolation of RNA. The prefiltration was performed with prefilter trays (Applied Biosystems). 350µl of each tissue lysate was pipetted into the wells and then a vaccum pressure of 80% was applied for 180s. The prefilter tray was then removed and the resulting filtered lysate was diluted to 50% in lysate buffer. The purification tray was pre-wet with 40µl of RNA Purification Wash solution 1 (Applied Biosystems). Then 350µl of the samples were pipetted into the wells of the purification tray and a vacuum pressure at 80% for 180 s was applied. The remaining lysate, 350µl, was then added and the same procedure was repeated. 400 µl of RNA Purfication Wash solution 1 was added to each well to perform the first wash, 80% for 180s. This step was repeated with 500 µl of RNA Purification Wash solution 2 (ethanol) (Applied Biosystems). To get rid of contaminating DNA 50 µl of DNAse (Absolute RNA wash solution, Applied Biosystems) was added and left to operate in the filters for 15 minutes. The third and fourth wash were also performed with RNA Purification Wash solution 2, 300µl, and a vacuum pressure at 60% was applied for 120 s. Then a vacuum pressure at 90% for 300 s was applied to dry the membrane. To elute, 100µl of Nucleic Acid Purification Elution Solution (Applied Biosystems) was added to each well followed by a pressure of 20% for two minutes. The RNA was then pipetted to RNase free Eppendorf tubes and kept at -80°C.

*RNeasy® Qiagen*

*Mini kit*

The first re-extractions were made with the same lysate i.e including the lysis buffer of Applied Biosystems. These were diluted to fifty percent in the lysis buffer included in the RNeasy® Mini kit (Qiagen) prior to extraction to give a total volume of 600-700 µl. The lysates were than added to Qiashredder spin columns (Qiagen) and centrifuged for two minutes at 12000 rpm. Thereafter one volume of 70% ethanol was added and the rest of the protocol was followed according to the manufacturer's instructions (animal tissues). Elution was made in 40-50 µl of water. The RNA were stored in RNase free Eppendorf tubes at -80 °C.

*Midi kit*

The first re-extractions were made with the same lysate i.e including the lysis buffer of Applied Biosystems. These were diluted to fifty percent in the lysis buffer included in the RNeasy® Midi kit (Qiagen) prior to extraction to give a total volume of 1.5-2 ml. The extractions were otherwise performed according to the manufacturer's protocol.

### 2.5 Analysis of the RNA quality

*Agilent 2100 Bioanalyzer*

Analysis was performed with the Agilent 2100 Bioanalyzer (Agilent technologies, Massy, France) and the RNA 6000 Nano Labchip® Kit (Agilent technologies). The gel included in the

kit was filtered by a centrifugation at 4000 rpm (2 minutes) in a spin filter and then a dye concentrate at RT was added, 1µl per 66µl gel. The mix was centrifuged at 14000 rpm for ten minutes to situate gel aggregates to the bottom of the tube. The gel was than pipetted to an RNA nano chip according to the manufacturer's directions. 5µl of the Nano marker was added to the 12 sample wells as well as to the ladder well. 2µl of each of the RNA samples and the ladder were then heat denatured in 70°C for 2 minutes to minimize secondary structure and the tubes rapidly centrifugated to spin down the droplets. 1µl of the ladder was loaded into the ladder well and 1µl of the samples pipetted into the 12 sample wells. The chip was vortexed in an IKA vortexer for one minute and then directly put into the Agilent 2100 bioanalyzer. An Eukaryote Total RNA Nano assay was run and the results were presented as electropherograms and gel-like images. After a successful run, the results of the ladder well included 6 RNA peaks and 1 marker peak and the results of the sample wells 2 ribosomal peaks (18S and 28S) and the marker peak.

### 2.6 cDNA

*High capacity cDNA Archive kit (Applied Biosystems)*

For the reverse transcription the High Capacity cDNA Archive kit (Applied Biosystems) was used. The cDNAs were prepared in series of 20-30 at a time. The reagents and the RNA were thawed on ice. The mastermix for a series of samples was prepared. For each sample the mastermix contained 10µl of reverse transcription buffer (10x), 4µl of dNTPs (25x), 10µl of random primers (10x), 5µl of multiscribe reverse transcriptase (RT) and 5 µl of RNase inhibitor, giving 34 µl of mastermix/sample. The mastermix was added to PCR tubes kept on ice. The cDNAs were prepared as to produce the same amount of DNA for each sample i.e. 4µg so the volume of RNA needed to give 4µg was calculated and pipetted into the mastermix and finally sterile water was added to give a total volume of 100µl. The tubes were briefly centrifuged to spin down the contents and to eliminate air bubbles. The reverse transcription was performed in the Thermal cycler Primus 96 (MWG AG Biotech) and the program used included 10 minutes at 25°C followed by two hours at 37°C. For the PCR reaction 5 µl of every cDNA was put in tubes and diluted to 50 µl, theoretically giving a concentration of 4ng/µl. The cDNAs were kept at –80°C until the real time quantitative PCR.

### 2.7 Real-time quantitative RT-PCR

*Taqman® Low Density Array*

The low density arrays (Applied Biosystems) included 48 chosen genes (of which one was dysfunctional; primers and/or probes were not working), among these housekeeping genes and genes of interest. The 384 wells of each card comprised primers and probes. A passive reference called ROX™ was also present. For each sample a mix consisting of 25µl of cDNA (100ng), 25 µl of sterile water and 50 µl of mastermix (TaqMan® Fast Universal Master Mix, Applied Biosystems) was prepared in eppendorf tubes. The resulting 100µl were then loaded into the reservoirs of the low density arrays, 8 samples/card. To distribute these 100 µl into each of the 48 wells the cards were centrifuged in a standard Legend T centrifuge (Sorvall) for 2x1 min at 1200 rpm (up ramp and down ramp at 9). Following the centrifugation the interconnecting channels between the wells were occluded and the reservoirs were cut off. The low density arrays were then loaded into the ABI Prism® 7900HT Fast Real-Time PCR System (Applied Biosystems) and the program started; 40 cycles (2 min at 50°C, 10 min at 94.5°C, 30 s at 97°C and 1 min at 59.7°C). Two samples were analysed as duplicates and the remaining samples as single samples.

*Calculations with the obtained data*

The exponential amplification of a target in a PCR is described by the equation

$$X_n = X_0 \times (1 + E_x)^n \quad \text{(i)}$$

*Where $X_n$ is the number of target molecules at cycle n, $X_0$ is the initial number of target molecules, $E_x$ the efficiency of target amplification and n the number of cycles.*

The threshold cycle (Ct) indicates the fractional cycle number at which the amount of amplified target reaches a fixed threshold, and (i) can be re-written to

$$X_T = X_0 \times (1 + E_x)^{Ct,n} = KX \quad \text{(ii)}$$

*Where $X_T$ is the number of target molecules at the threshold cycle, $Ct_X$ the threshold cycle for target amplification and KX a constant.*

Similarly, the equation for an endogenous reference is described by

$$R_T = R_0 \times (1 + E_R)^{Ct,r} = KR \quad \text{(iii)}$$

*Where $R_T$ is the number of reference molecules at the threshold, $R_0$ the initial number of reference molecules, $E_R$ the efficiency of the reference amplification and KR a constant.*

Dividing $X_T$ by $R_T$ gives the expression

$$\frac{X_T}{R_T} = \left( \frac{X_0 \times (1 + E_X)^{Ct,x}}{R_0 \times (1 + E_R)^{Ct,r}} \right) \quad \text{(iv)}$$

The efficiencies of the amplifications of the targets and the references are assumed to be equal, $E_X=E_R=E$, and therefore (iv) can be transformed to

$$\frac{X_T}{R_T} = \frac{X_0}{R_0} \times (1 + E)^{Ct,x - Ct,r} = K \quad \text{(v)}$$

or

$$X_N \times (1 + E)^{\Delta Ct} = K \quad \text{(vi)}$$

*Where $X_N$ is the normalised amount of target and $\Delta Ct$ the difference in threshold cycles between target and reference.*

(vi) can be rearranged to

$$X_N = K \times (1 + E)^{-\Delta Ct} \quad \text{(vii)}$$

Finally $X_N$ for any sample *m* is divided by $X_N$ for the calibrator *cb*

$$\frac{X_{N,m}}{X_{N,cb}} = \frac{K \times (1 + E)^{-\Delta Ct,m}}{K \times (1 + E)^{-\Delta Ct,cb}} = (1 + E)^{-\Delta Ct,m + \Delta Ct,cb} = (1 + E)^{-\Delta\Delta Ct} \quad \text{(viii)}$$

The efficiency is approximated to 1 (one assumes that the amount of target molecule doubles at every cycle) and the amount of target normalised to a reference and a calibrator sample is given by

$$\frac{X_{N,m}}{X_{N,cb}} = 2^{-\Delta\Delta Ct} \quad \text{(ix)}$$

For the $2^{-\Delta\Delta Ct}$ method to be valid the amplification efficiency of the target and the reference must be approximately equal[7]. The purpose of the reference gene is to normalise for the amount of cDNA added to the wells of the low density arrays.[13]

# RESULTS

The aim with this work was to explore the presence of immune factors in the form of mRNA and look at the correlation with the micrometastatic state. 76 colon tumour specimens were investigated. Extraction of RNA from cancerous tissues was followed by retrotranscription of RNA to cDNA. The expression level of 47 genes was then determined with quantitative PCR.

## 3.1 Comparative analysis of two RNA extraction kits

The extractions of RNA were initially performed with the ABI Prism™ 6100 Nucleic Acid PrepStation (Applied Biosystems). The homogenized tissue loaded to each well corresponded to 8-10 mg. There were a great number of clogged wells during the purification process. An assay was performed with less tissue, 5-7 mg, and the clogging was indeed avoided but the RNA was of too low concentration. Therefore about 10 mg of the whole series of tumours were extracted. The clogged wells amounted to about a third of the total number of wells. The RNA were analysed with the Agilent 2100 Bioanalyzer, which showed that no or degraded RNA were obtained from the clogged wells and revealed a bad quality of many of the RNA extracted from the other wells. The Bioanalyzer results of a non-degraded RNA should exhibit 2 ribosomal peaks (18S and 28S) with a ratio 28S/18S preferentially above 2. The RNA exhibiting two clearly distinguishable peaks, and a ratio close to or above 0.9 was initially considered acceptable. As can be seen in table 3.1, the consequence of the clogged wells and the mediocre quality of the extractions was that useful RNA were obtained only from 41 % of the original samples. The visual difference between acceptable and degraded RNA is showed in figure 3.1.

| **Résumé of RNA extraction with the ABI Prism** | n° | % |
|---|---|---|
| Homogenized samples | 169 | 100 % |
| Non-clogged wells | 109 | 64 % |
| RNA of decent quality in non-clogged wells | 70 | 41 % |

**Table 3.1** Results of the RNA extractions with the ABI prism™ 6100 Nucleic Acid PrepStation. n° stands for the number of samples and % the corresponding percentage.

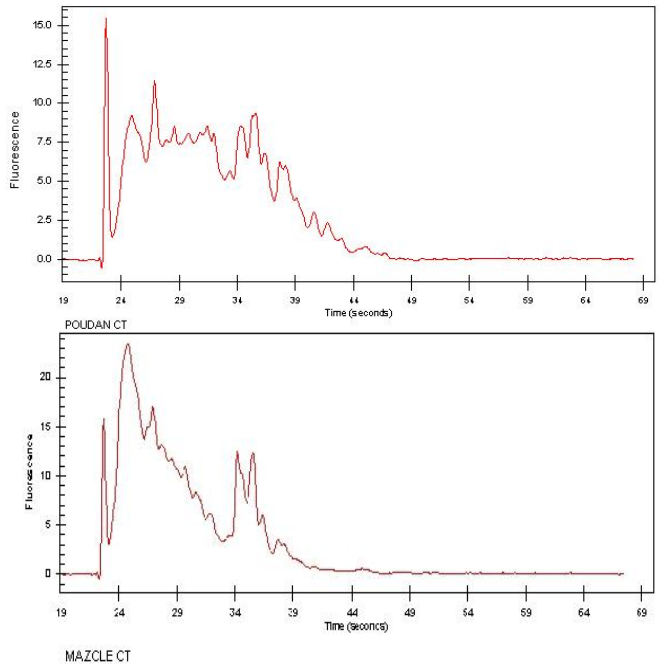
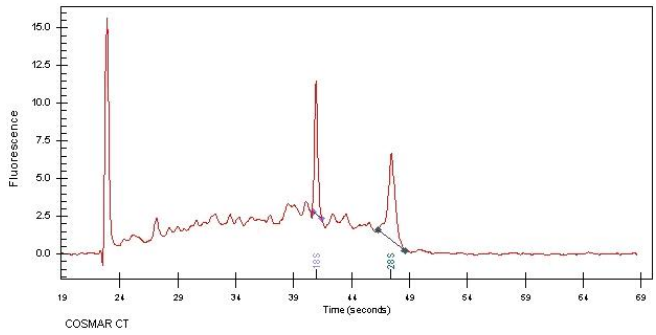



**Figure 3.1** Examples of RNA extracted with the ABI Prism™
A) POUDAN (CT: colon tumour sample)
B) MAZCLE (CT)
C) COSMAR (CT)

A) The resulting extract from a lysate that clogged a well. No ratio could be calculated since no 18S/28S peaks were visible.

B) Example of a degraded RNA obtained from a non-clogged well. No ratio could be calculated since no 18S/28S peaks were visible.




C) Example of an extracted RNA where the two ribosomal peaks, 18S and 28S, were visible after extraction. The ratio obtained was 1,35, however one can see that the RNA is slightly degraded from the irregular baseline and the fact that the 28S peak is lower than the 18S peak. The RNA was obtained from a non-clogged well.

The quality of the extraction kit was initially validated by extracting RNA from cellular lineages (data not shown). To verify if the variable quality of the RNA extracted from the cancerous tissues was due to heterogeneous quality of the tumour specimens or to an extraction method inefficient for colon tissues, an alternative extraction method was tested on some of the tumours. The kit chosen for this purpose was the RNeasy® Midi kit (Qiagen). The lysates selected for this test represented the range of quality obtained with the ABI Prism™:

- four lysates that had clogged the wells of the ABI Prism™ purification tray
- two lysates that were degraded although obtained from non clogged wells
- one lysate from which an RNA was successfully extracted with the ABI Prism™.

The overall quality of the RNA extracted with the RNeasy® Midi kit was clearly superior to the preceding extractions. The RNA had 28S/18S ratios of 1.3-1.6. These results showed that the cancerous tissues were of satisfactory quality.

An illustration of the quality of the extracted RNA according to the technique used for the extraction is showed in figure 3.4 for three tumours.

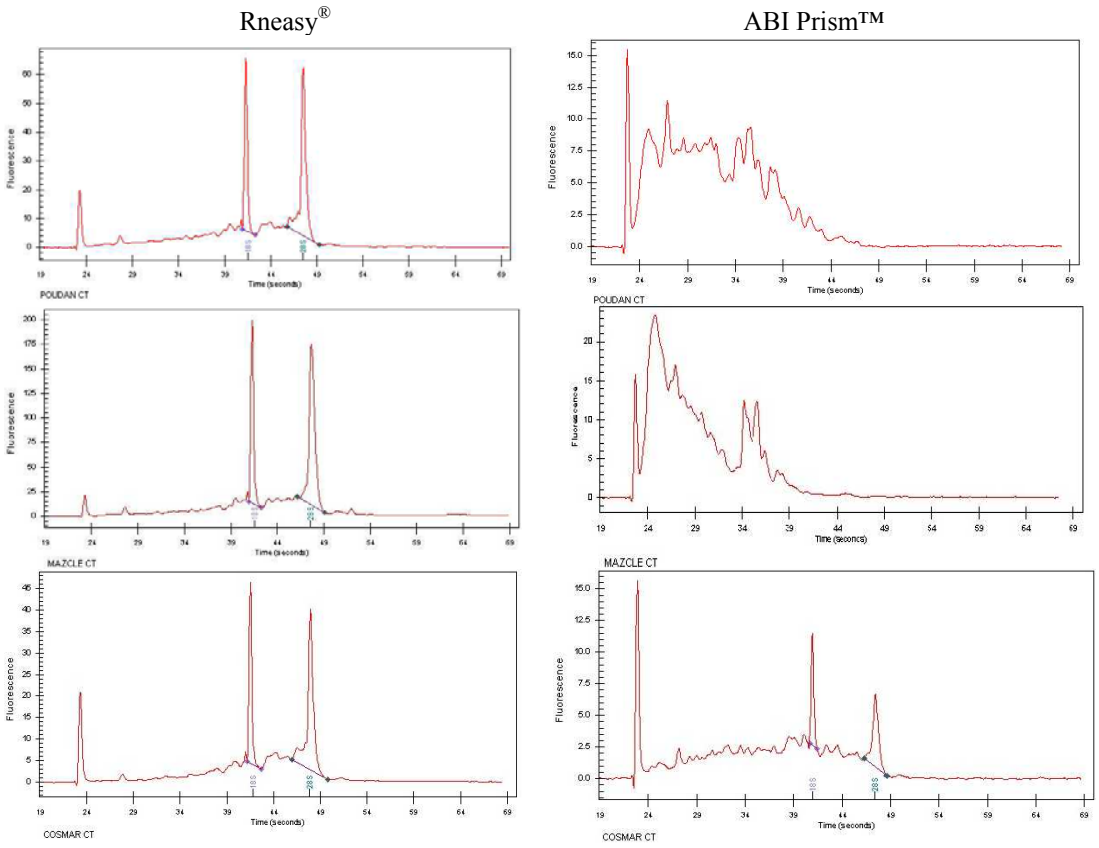



**Figure 3.4** Profiles of RNA extracted from three samples with the two kits, extractions with the RNeasy® Midi kit to the left and with the ABI Prism™ to the right. The samples are the same as in picture 3.1 i.e. POUDAN (CT) at the top, MAZCLE (CT) in the middle and COSMAR (CT) at the bottom.The RNA extracted with RNeasy® resulted in RNA of superior quality although the same lysates were used. The top picture on the right shows the resulting RNA from a clogged well and the others are from non-clogged wells. The ratio of the only RNA extracted with ABI Prism™ that exhibits two peaks is 1.35. The ratios of the RNA extracted with RNeasy® are (to the left) from the top down:1.49, 1.52 and 1.39.

The extractions that had resulted in degraded RNA or clogged wells were hence remade with the RNeasy® Midi kit. For the samples where only a small volume of lysate was left (<200µl) the extraction was made with the RNeasy® Mini kit. The results from the RNA preparations are summarised in table 3.2. The complete series extracted with RNeasy® included 145 samples and resulted in 110 decent RNA (76%). The extracted quantities were also more substantial with RNeasy® as compared with the ABI preparation method.

| **Extraction method** | ABI Prism™ (Applied Biosystems) | RNeasy® (Qiagen) |
|---|---|---|
| **Median 28S/18S ratio** | 0.8 | 1.2 |
| **Average quantity extracted (ug)** | 17 | 27 |

**Table 3.2** Comparison of the RNA resulting from the non-clogged wells of the ABI Prism™ and the samples extracted with RNeasy®.

Given that there was a slight quality difference between the RNA successfully extracted with the ABI Prism™ and the RNA extracted with RNeasy®, a quantitative PCR assay was made to elucidate whether these RNA could be used in the same PCR assays. Included were 10 RNA where the same lysate (i.e. 5 lysates) had been extracted with the two methods and 6 RNA extracted with the RNeasy® where the 28S/18S ratio was below 1.2.

The PCR assay was made with the genes *18S*, *FOX-P3* and *GAPDH* in duplicate with the ABI PRISM 7900HT System. The genes were chosen to represent a wide expression range. *18S* and *GAPDH* are housekeeping genes, *18S* is expressed at a very high level and *GAPDH* at a high level. *FOX-P3*, which is an immunologic gene associated with the regulatory phenotype of T cells, is usually weakly expressed in tumour specimens. The same amount of RNA was reversely transcribed for all the samples.

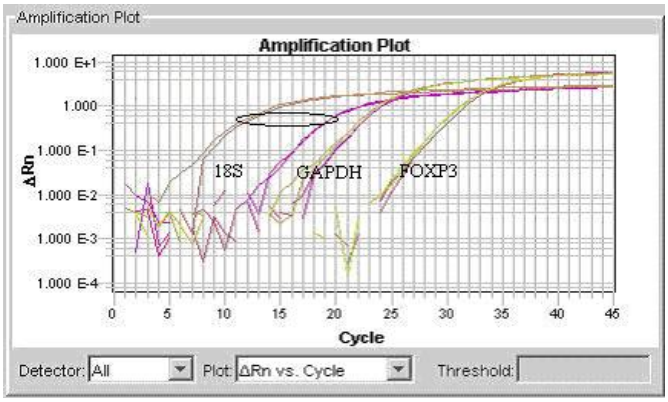



**Figure 3.5** An RNA extracted with ABI and an RNA extracted with RNeasy, both from the same lysate.The RNA extracted with ABI with a ratio of 0.77 and the RNA extracted with RNeasy with a ratio of 1.34. The genes are in the order *18S*, *GAPDH* and FOXP3 and the sample extracted with ABI is in pink and the sample extracted with Rneasy® is in brown. All samples were made in duplicate.

The results showed a significant difference in 18S expression when comparing samples extracted with the two different methods (example in figure 3.5). The samples extracted with ABI Prism™ had a lower concentration of 18S (the pink curve in figure 3.5). The expressions for the other two genes were similar for the samples irrespective of the extraction method. When comparing two RNA extracted with the RNeasy® kit even though their quality features as analysed with the Bioanalyzer were dissimilar (degraded versus not degraded) the curves of amplification of 18S were identical (figure 3.6). However, while the curves of 18S seem identical, the expression of GAPDH was lower and the expression of FOXp3 was higher for the very same samples (figure 3.6).

This comparison strongly suggested that only one extraction method should be used for the specimens included in the study. Therefore also all the good-quality RNA extracted with the ABI Prism™ (70 samples) were replaced by preparations made with RNeasy® Midi/Mini.

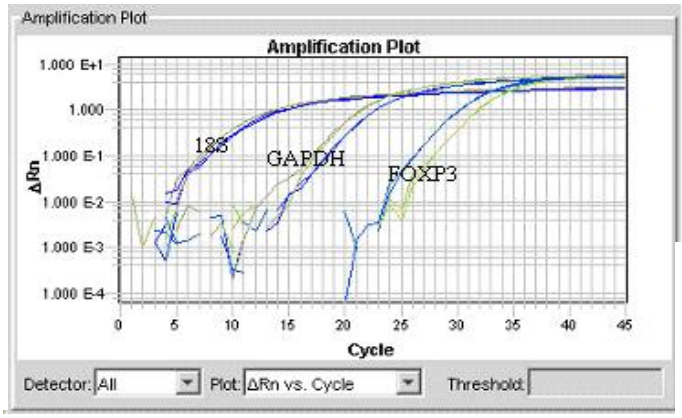



**Figure 3.6** Two RNA extracted with RNeasy®, the first partially degraded (ratio: 0,59 ) and the second with a ratio *28S*/*18S* of 1.67. The blue line represents the degraded RNA and the grey the RNA with ratio 1,67. The genes are in the order *18S*, *GAPDH* and FOXP3.

## 3.2 The cDNAs and genes included in the study

### 3.2.1 The selected cDNAs

The cDNAs included in the analyses with the low density arrays were retrotranscribed RNA extracted from (the detailed list in Appendix III):

- 76 colon tumours
- 7 couples tumour / liver metastase
- 6 colon samples from healthy tissue
- 5 tumoral lineages (HT29, SW620, CaCo2, LS174T, LOVO, ATCC origin)
- 1 mix of cDNA from colon tumours
- 1 mix of cDNA from normal colon tissues
- 1 mix of cDNA from liver metastases
- 9 samples with purified T lymphocytes

The mixes were prepared to be used in the normalisation step.

### 3.2.2 The selected genes

The 48 genes included in the arrays are listed in Appendix I. The genes were chosen to obtain profiles of every sample in the areas of activation, anergy, cytotoxicity and phenotypic orientation regarding $T_H1$ and $T_H2$. Two housekeeping genes were included for the normalisation step.

## 3.3 Quality control of the amplifications

When Applied Biosystems tested the low-density arrays before supplying them it was established that the TaqMan® probe for the *STAT1* gene was dysfunctional, i.e. it did not bind cDNA. In total with 14 low density arrays, 112 cDNAs were amplified for the 47 genes, resulting in 5264 real time quantitative PCR reactions. The PCRs in the remaining wells were carried out successfully; no other wells than those for *STAT1* were defect.

### 3.3.1 Reproducibility

Two samples were analysed in duplicate, the colon tumour mix cDNA and one of the colon tumour cDNA (OLIAME CT). The reproducibility can be viewed in figure 3.7 and 3.8. The remaining samples were analysed as single samples.

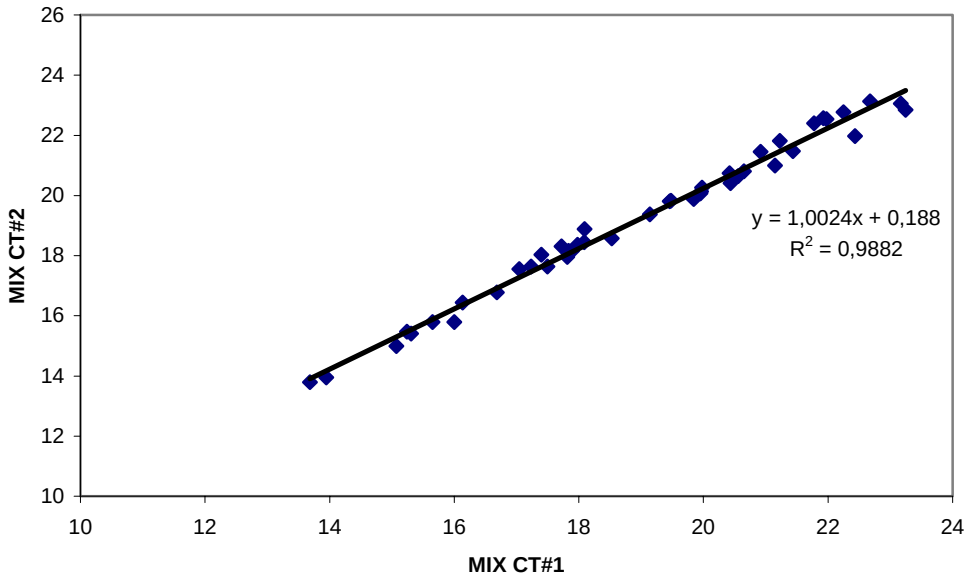



**Figure 3.7** Correlation plot of the duplicate MIX CT (colon tumour). The trend line is described by the equation y=1,0024x+0188 ($R^2$=0,9882).

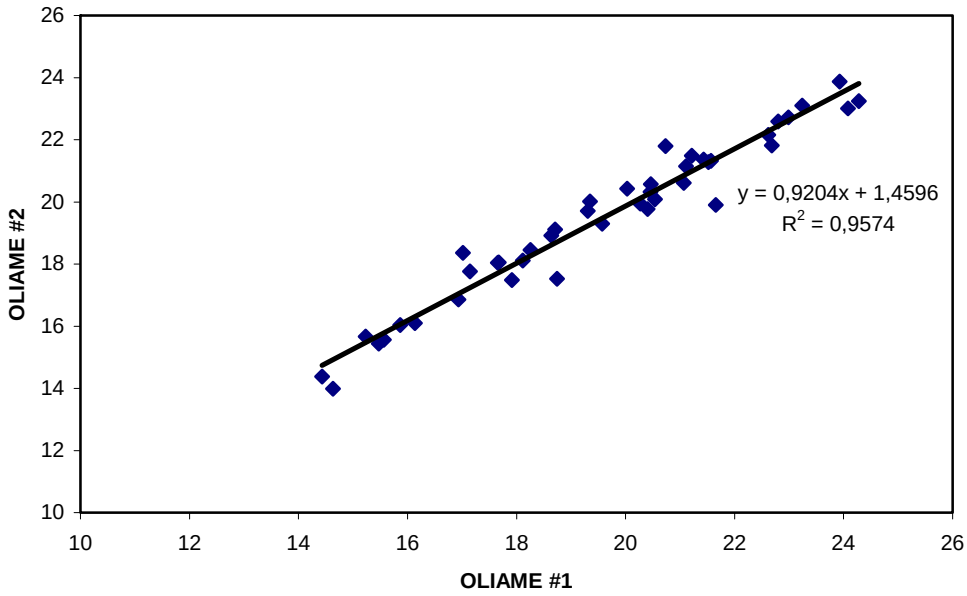



**Figure 3.8** Correlation plot of the duplicate OLIAME CT. The trend line is described by the equation y=1,0024x+1,456 ($R^2$=0,9574)

### 3.3.2 Homogeneity of the curves

The same amount of RNA was transcribed and loaded for every sample to the LDAs. One can observe from the amplification curves for 18S and GAPDH, in figure 3.9, that the curves seem more heterogenous for GAPDH.

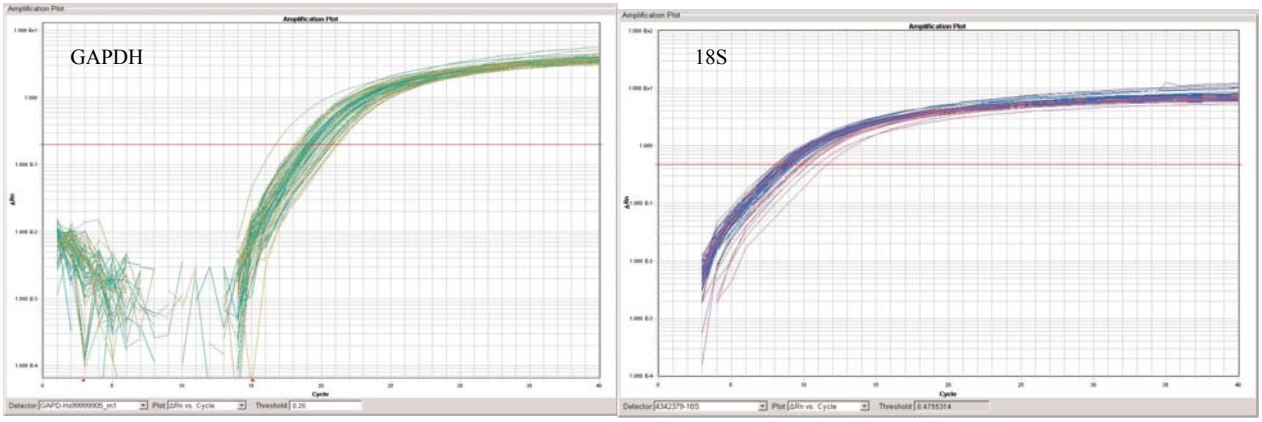



**Figure 3.9** Amplification curves of GAPDH, to the left, and 18S to the right.

The correlation plot between the cycle threshold (Ct) values of 18S and GAPDH is presented in figure 3.10A. Except for two samples there seems to be a rather good correlation between 18S and GAPDH. This can also be seen in figure 3.10b-c where the ΔCt values for CD8 and T-bet (TBX-21) are plotted (the two discordant samples were eliminated in these graphs). The two deviant samples were excluded from the results analysis.

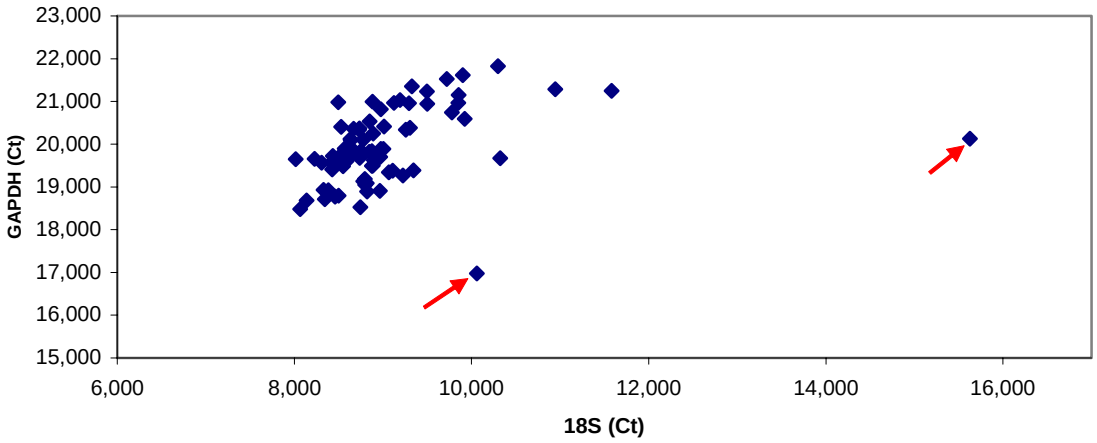



**Figure 3.10A** Correlation plot of 18S and GAPDH. $R^2$=0,1139. The two samples most distant from the others are LESMAR and SIGGEN.

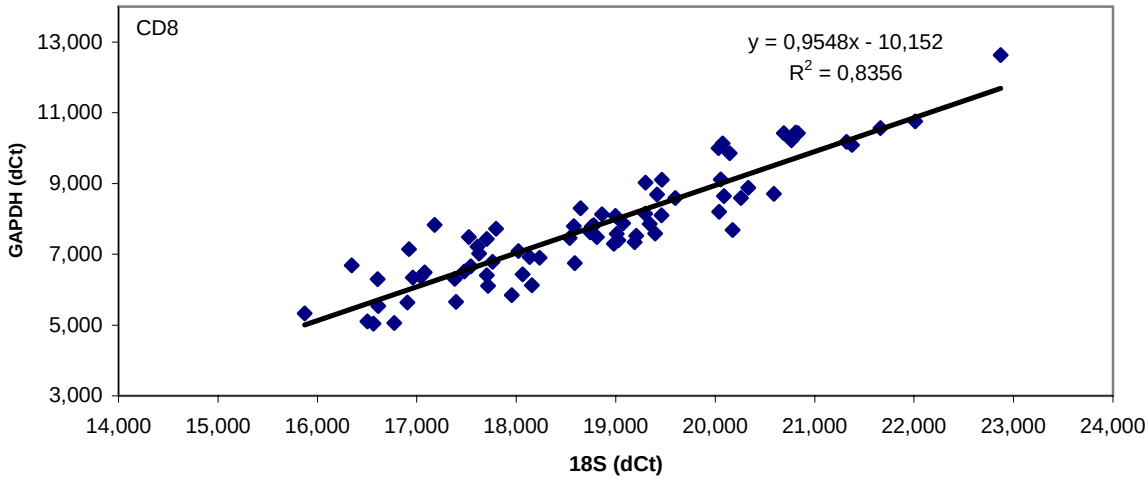



**Figure 3.10b** Correlation plot of CD8 dCt values for 18S and GAPDH. When the two discording samples from figure 3.10a were removed the trendline had the equation y = 0,9548x – 10,152, $R^2$=0,8356.

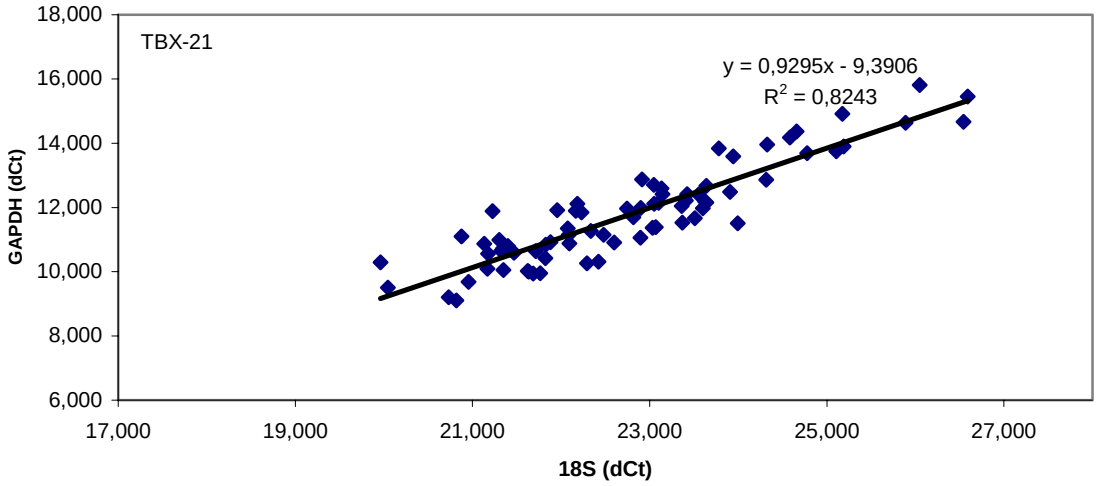



**Figure 3.10c** Correlation plot of TBX-21 dCt values for 18S and GAPDH. When the two samples distant from the others were removed the trendline had the equation y = 0,9295x – 9,3906, $R^2$=0,8243.

The average Ct values of 18S and GAPDH for normal colon tissues, tumoral colon tissues and tumoral liver tissues are shown in figure 3.11. The variability for 18S (but not for GAPDH) seems to be important in the liver samples, which suggests that there is variability according to tissue type. Before further analysis on the liver specimens these results should be confirmed since only 7 single samples were investigated in this study. For every type of tissue the homogeneity of 18S and GAPDH should be evaluated to find the best reference gene.

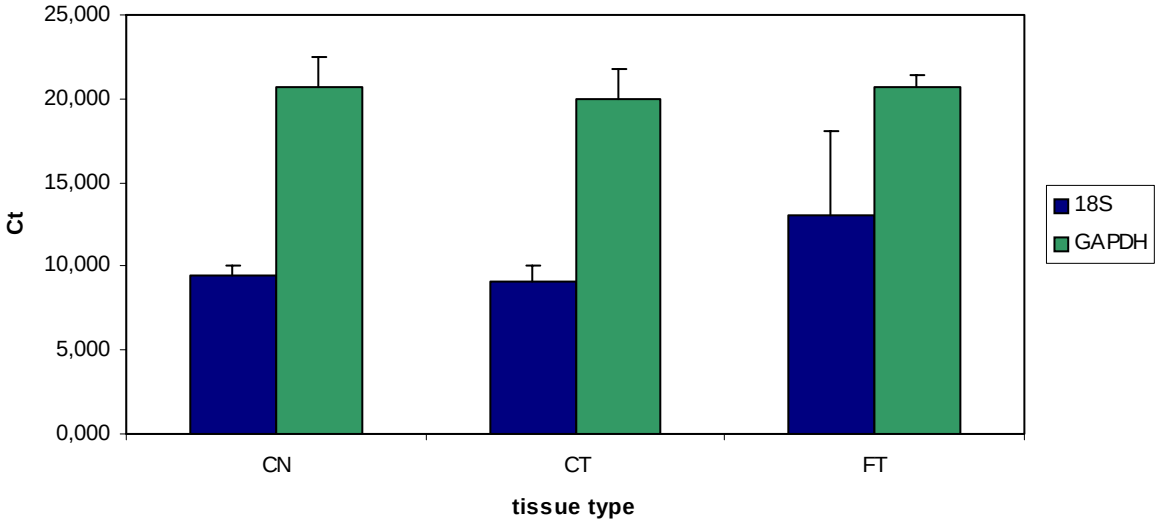



**Figure 3.11** Average Ct values for *18S* and *GAPDH* with standard deviation determined in normal colon (CN), cancerous colon (CT) and cancerous liver tissues (FT).

## 3.5 Control of immunologic profiles observed with low density arrays

Samples with purified T lymphocytes were included in this study, which allowed for a quality control. Figure 3.12 shows the relative expression of T lymphocytes stimulated with IL-2 (and with antibodies against CD3 and CD28) calibrated to non-stimulated T lymphocytes (normalisation to 18S). A number of genes included in the study are expressed by T lymphocytes. After stimulation the expression of some of these increases. The stimulation leads to a $T_H1$ defence, and the expression of IFN-γ, IRF1 and TBX-21 all increase due to this

stimulation, which is expected. GATA-3, on the other hand, involved in $T_H2$ defence decreases.

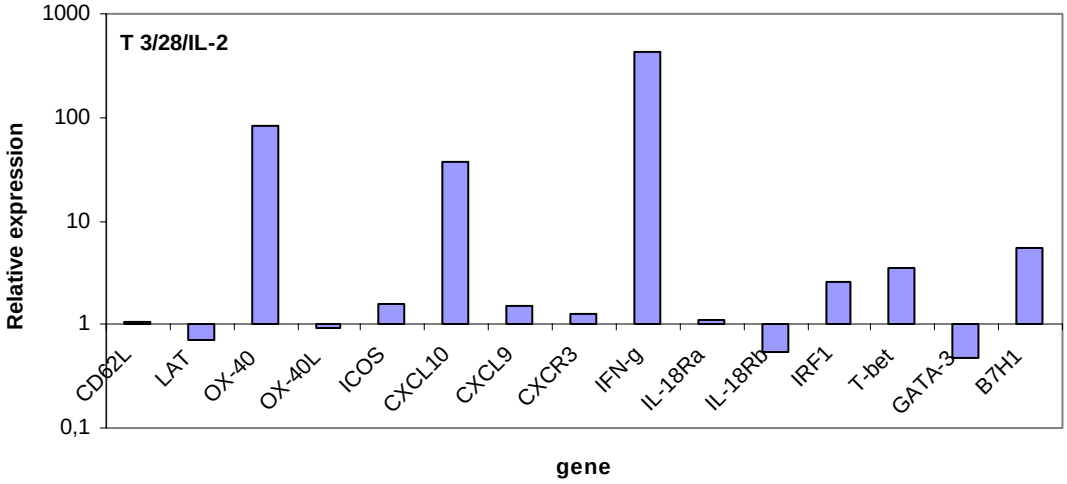



**Figure 3.12** The relative expression of T lymphocytes stimulated with IL-2 in comparison with non-stimulated T lymphocytes. The genes CD62L-ICOS are involved in activation, CXCL10-GATA-3 in defense orientation and B7H3 in anergy.

Figure 3.13A shows that the tumoral lineage SW480 expressed the genes related to tumoral expression of colon cancer included in the LDAs. SW480 is a lineage derived from a colon cancer. SW620 is the metastatic cell line associated to the same colon cancer. Figure 3.13B shows the relative expression of SW620 calibrated to SW480. The transcriptional profile shows that there are a few differences in expression. The immunologic genes are weakly expressed in these lineages, for instance CD3 and IFN-γ were not detected at all (data not shown).

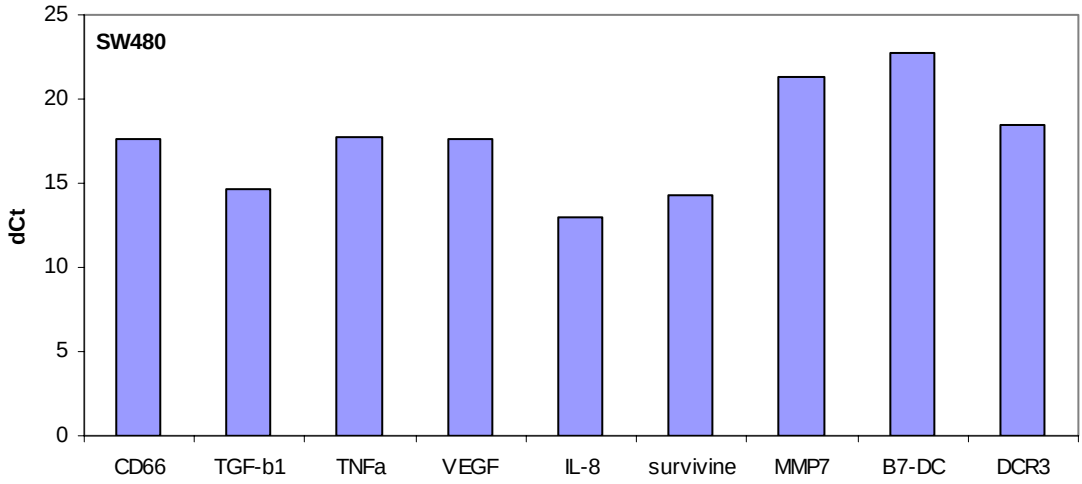



**Figure 3.13A** The expression by the tumoral lineage SW480 of the genes included in the LDAs related to tumoral expression in colon cancer. Note that since the result is presented as dCt values ($Ct^{gene}$-$Ct^{18S}$), a higher value means lower expression.

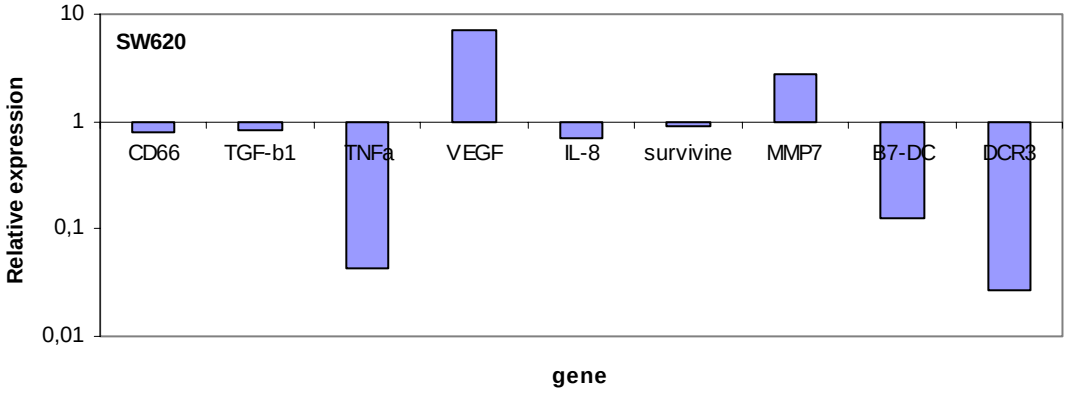


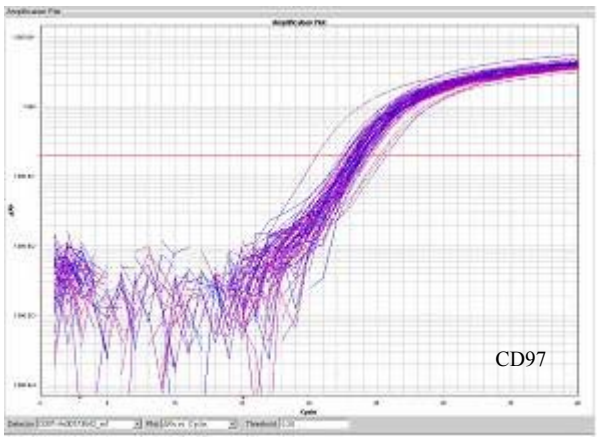

**Figure 3.13B** Relative expression in the tumoral lineage SW620 in comparison to SW480. (Normalisation was made to 18S, SW480 was the calibrator.)

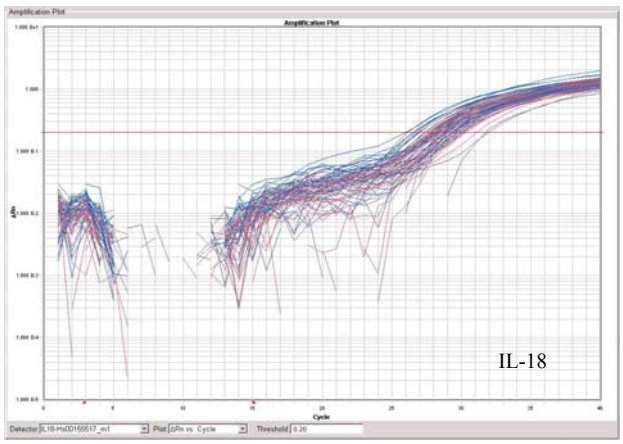
## 3.6 Calculations with the obtained data

The acquired data were analysed by relative quantification with the $2^{-\Delta\Delta Ct}$ method as recommended by Applied Biosystems. The data are, with this method, presented as the fold change in gene expression normalised to an endogenous reference gene and to a calibrator sample. The data from the analysis were transferred to the SDS 2.2 software and subsequently exported to Microsoft Excel for interpretation. For the $2^{-\Delta\Delta Ct}$ method to be valid the amplification efficiency of the target and the reference must be approximately equal. The purpose of the reference gene is to normalise for the amount of cDNA added to the wells of the low density arrays.

### 3.6.1 Amplification curves

Most amplification curves had quite similar appearances. The positioning of the threshold was done automatically by the SDS software. It is important that the threshold is positioned in the exponential phase of the curves. Only the threshold for IL-18 was repositioned manually, the SDS software did not manage to put it right probably due to an amplification curve that had a less steep slope (figure 3.14). A few curves presented profiles quite different from the others (for example granzyme B figure 3.14). The appearances of some different curves can be seen in figure 3.14.



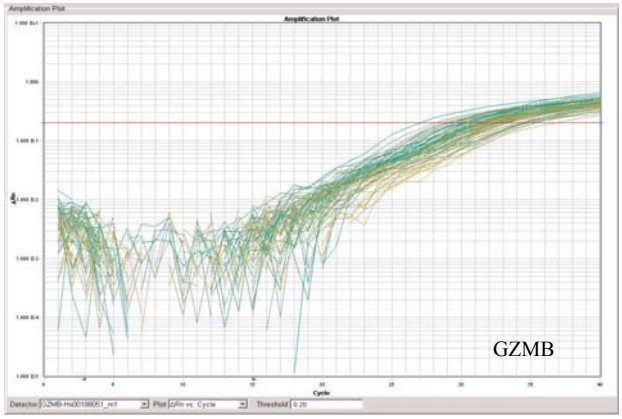



**Figure 3.14** Amplification profiles of CD97, IL-18 and granzyme B (GZMB).

Thus, the shape of certain curves, such as those for granzyme B (GZMB), in comparison with, for instance, those for 18S is quite dissimilar. This could indicate a variable efficiency of the amplifications for different PCR assays.

### 3.6.2 Preliminary results

The values exported from SDS 2.2 included ΔCt values for every sample (normalised with the amount of 18S) for a selection of the genes included in the study. The median ΔCt was determined for the patient groups of interest. The groups are listed in table 3.3. These median ΔCt were used as calibrators to calculate ΔΔCt. Then the value of $2^{-\Delta\Delta Ct}$ was determined and was represented as log10. Graphics (histograms) were created for all individuals belonging to the V-L-P- group calibrated with the median of the V+L+P+ group and all individuals belonging to the V+L+P+ group calibrated with the median of the V-L-P- group. The results presented include all patients except two, LESMAR and SIGGEN, who appeared to have inhomogenous Ct.

| **Patient groups** | **Micrometastatic state** |
|---|---|
| V-L-P- | Patients without micrometastases |
| V+ L+ P+ | Patients with micrometastases in blood vessels and/or in lymphatic vessels and/or along perineural structures |

**Table 3.3** The different patient groups.

***CD8*** 56% (27/48) of the V-L-P- patients had a higher expression of CD8 than the patients with micrometastases (figure 3.15). The expression of CD8 among the patients with higher expression than the calibrator varied between 1.0 times (patient 38 in the graph; DEBTHE) and 8.1 times higher (patient 42 in the graph, NASEST) than the calibrator. The average expression of CD8 in the group of the 27 patients was 3.0 times superior to the V+L+P+ group (the standard deviation, SD, was 1.7).

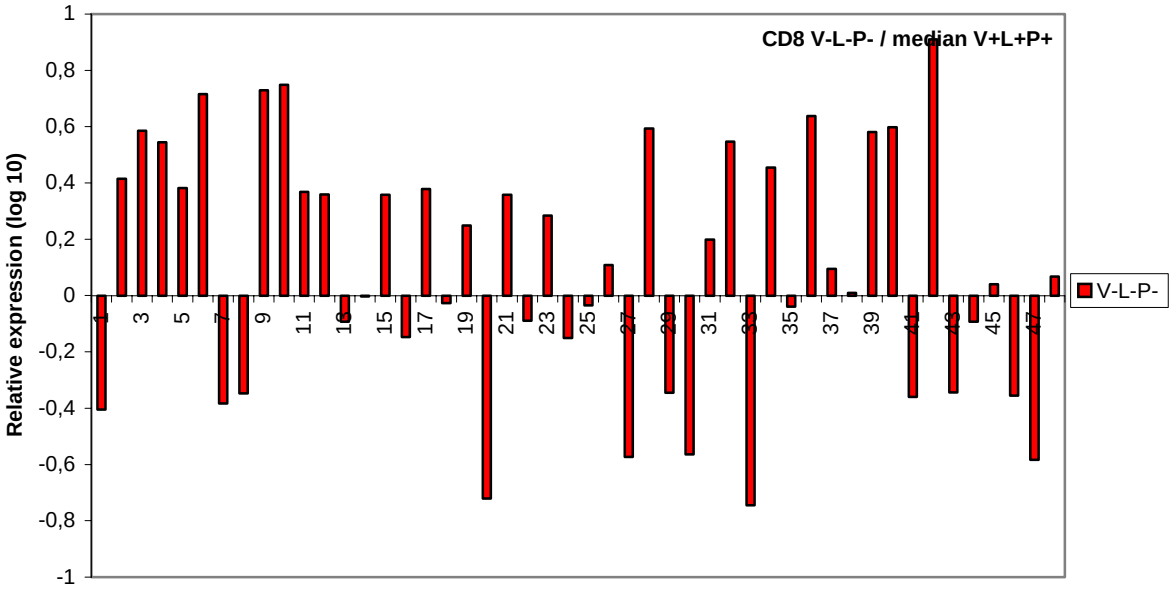



**Figure 3.15** Expression pattern of CD8 in V-L-P- patients. The results are presented as log 10 numbers. An y-value of 1 corresponds to an expression 10 times higher than the calibrator.

Figure 3.16 is the resulting graph of expression comparisons of samples from patients with some form(s) of micrometastase(s) calibrated with the median of the patients without any micrometastases. Here 63% (15/24) of the patients had a lower expression than the V-L-P- calibrator. Among the 15 patients the expression varied between 17.8 times lower (patient 21 in the graph; UEDKOJ) and 1.0 time lower (patient 23 in the graph; HALKIR) with an average expression 3.6 times inferior to that of the V-L-P- group (the standard deviation, SD, was 4.6). An infiltrate rich in CD8 expressing cells estimated by the quantity of mRNA could be correlated to the micrometastatic state.

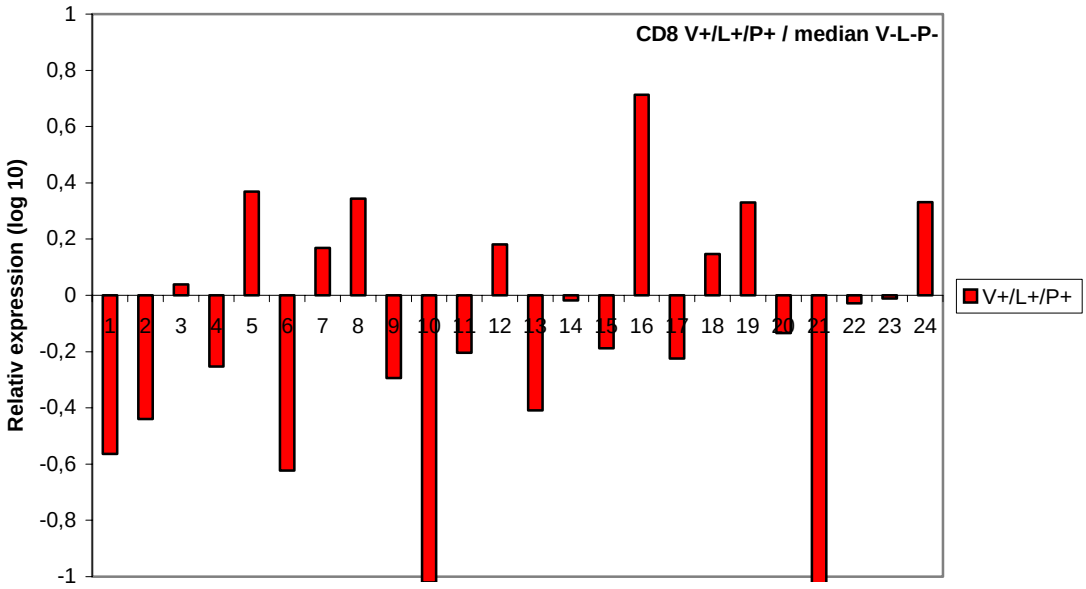



**Figure 3.16** Expression pattern of CD8 in V+L+P+ patients. Patient 10 has a value of $10^{-1,02}$ and patient 21 has a value of $10^{-1,25}$.

***CD4*** 65% (31/48) of the V-L-P- patients had a higher expression than the calibrator (figure 3.17). Highest expression among the 65% had patient 4 in the graph, THOJEA, with an expression 4.0 times stronger and lowest has patient 15, HEIYVE, with an expression 1.1 times higher than the calibrator. The average expression of CD4 in the group with an expression superior to the calibrator was 1.6 times stronger than that of the calibrator (SD 0.7).

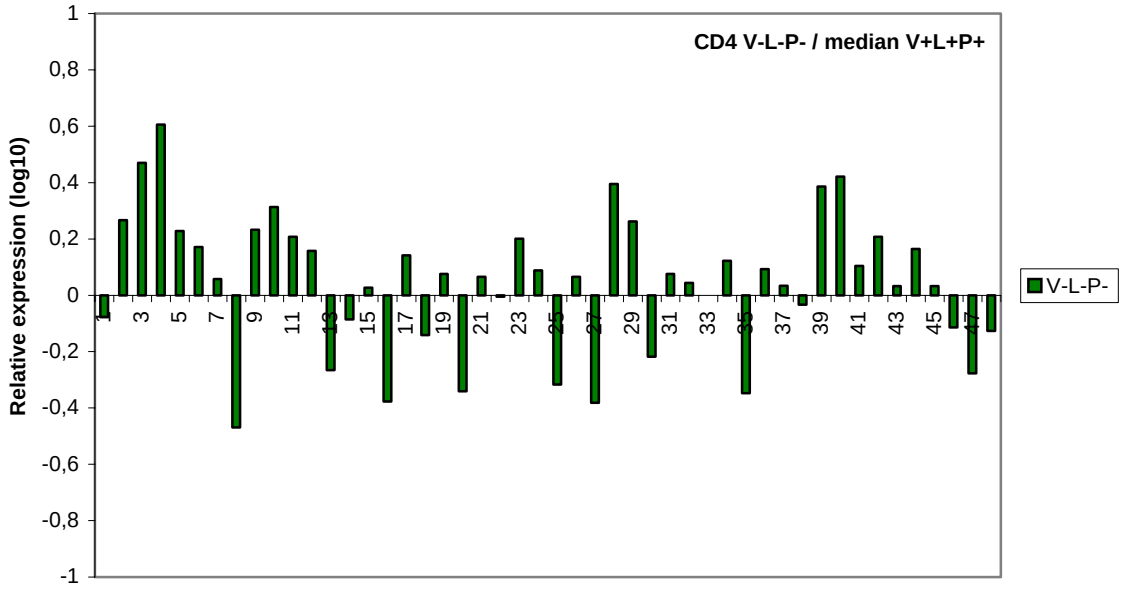



**Figure 3.17** Expression pattern of CD4 in V-L-P- patients.

When the expression patterns of V+L+P+ patients were calibrated to the patients negative for micrometastases 63% (15/24) had lower expression of CD4 than the calibrator (figure 3.18). The expression varied between 3.8 times lower (patient 21,UEDKOJ) and 1.0 times lower (patient 24, DAVDAN) than the calibrator in this group. In average the group with lower expression than the calibrator had an expression 1.8 times lower than the median of the V-L-P- group (SD 0.8).

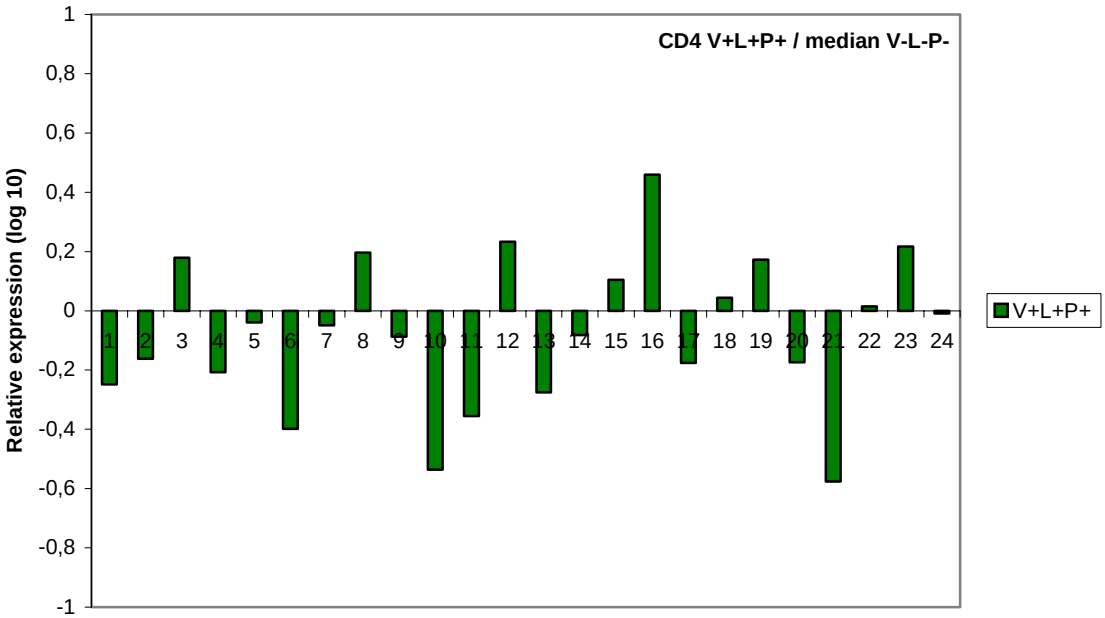



**Figure 3.18** Expression pattern of CD4 in V+L+P+ patients.

***T-bet (TBX-21)*** The T subtype signature in the patients was mostly investigated with genes associated to $T_H1$. The results for T-bet showed that its expression seemed to be more important in patients without any micrometastases, suggesting that a $T_H1$ profile could be associated with a better control of the micrometastatic process. 63% (30/48) of the V-L-P- samples had a higher expression than the calibrator (figure 3.19), the average expression within this group of 30 samples was 3.1 (SD 1.59) times stronger than the calibrator. The n-fold in this same group varied between 1.08 (patient 43; NDONDO) and 7.9 (patient 42; NASEST).

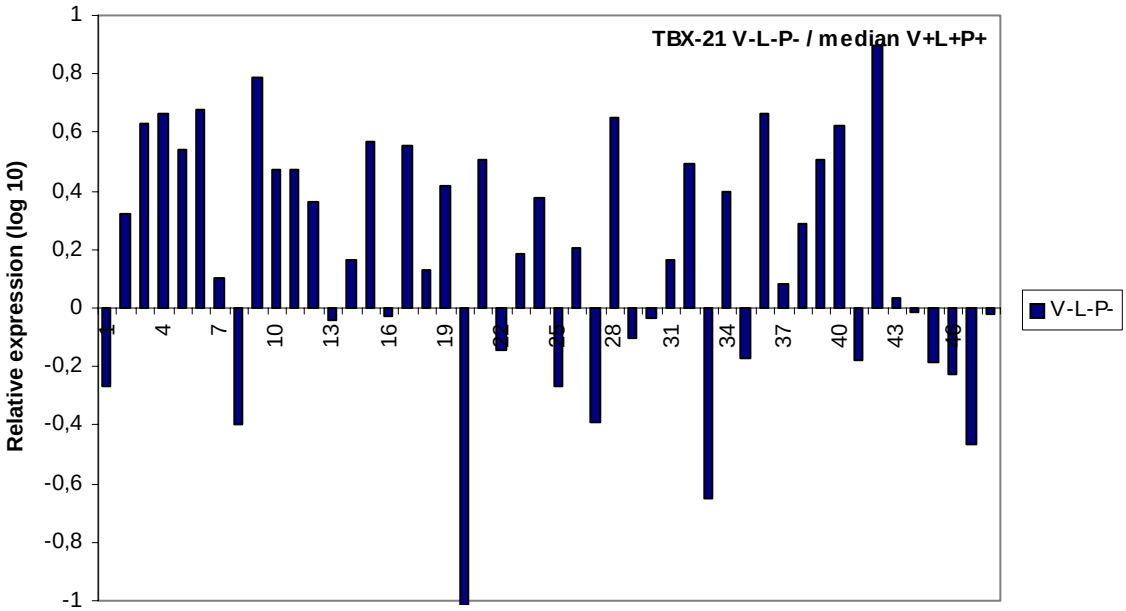



**Figure 3.19** Expression pattern of TBX21 in V-L-P- patients. Patient 20 has a value of $10^{-1,07}$.

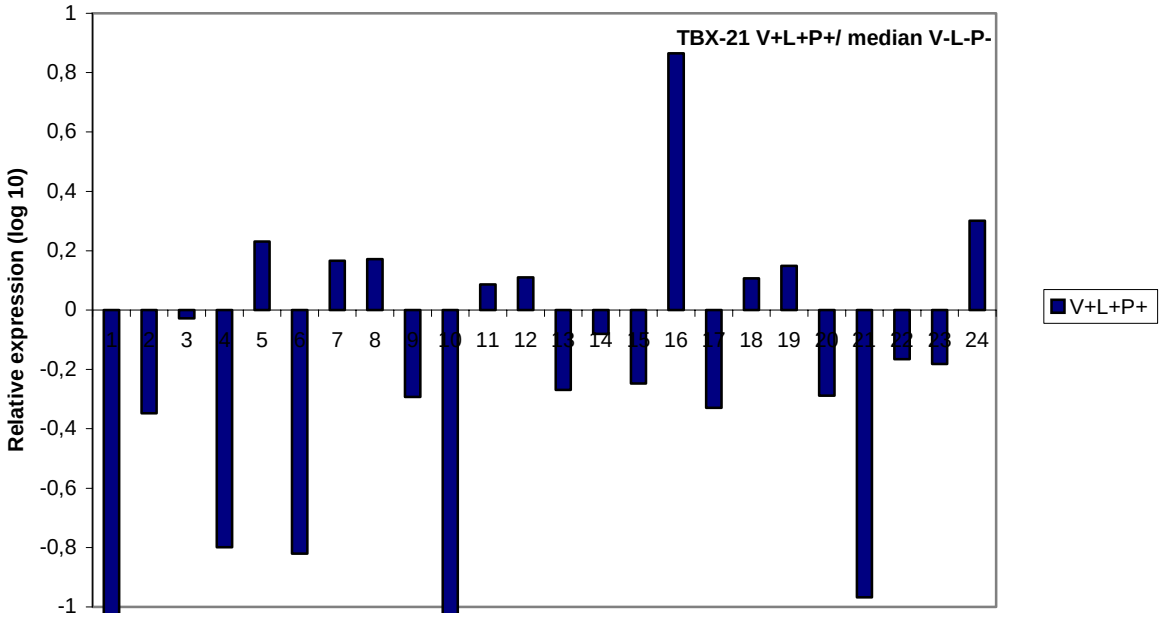



**Figure 3.20** Expression pattern of TBX-21 in V+L+P+ patients. Patient 1 has a value of $10^{-1,12}$ and patient 11 of $10^{-1,04}$.

When V+L+P+ patients were calibrated to the median V-L-P- patient (figure 3.20) 63% (15/24) had lower expression of T-bet than the calibrator. The expression among these 15 patients varied between 13.2 times lower (patient 1, BOUPAT) and 1.1 times lower (patient 3, SARALE) than the calibrator. The group with an expression inferior to the calibrator had in average an expression 4.2 times lower than the median of the V-L-P- group (SD 4.01). The results of IFN-γ, which is induced by T-bet, could confirm these results.

***IFN-γ*** The distribution of this cytokine in the group with no micrometastases calibrated with the positive group (figure 3.21) presented 30 patients (63%) with a higher expression of IFN-γ than the calibrator. The expression in the group with an expression superior to the calibrator's was spanning between 1.1 (patient 25, FOUMAR) and 22 (patient 42, NASEST) compared to the calibrator sample. The average expression of this group was 5.3 times stronger than for the calibrator (SD 5.0).

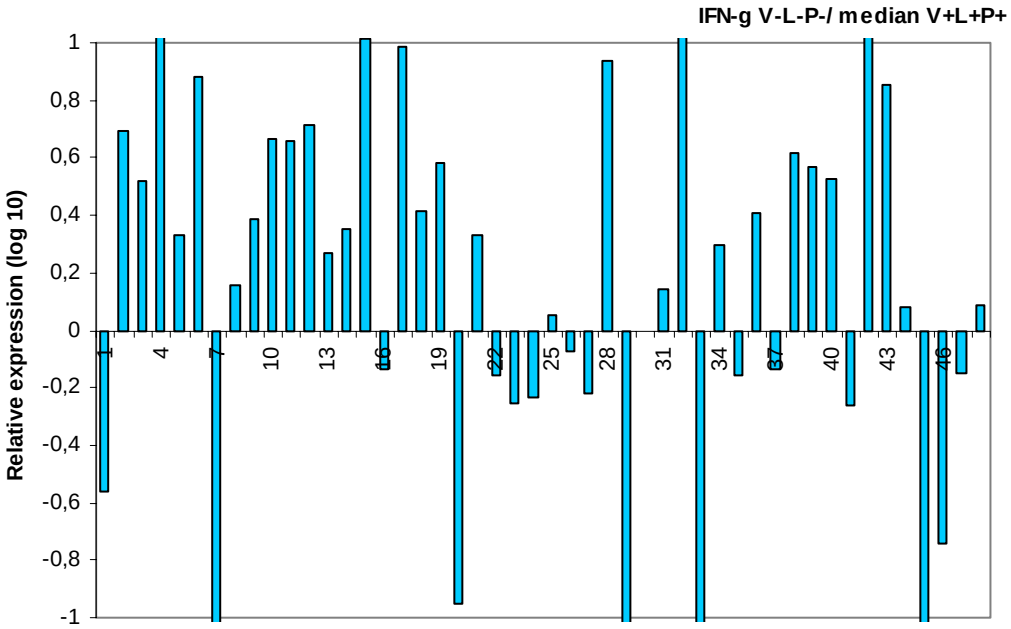



**Figure 3.21** Expression pattern of IFNg in V-L-P- patients. Patient 4 has a value of $10^{1,15}$, patient15 of $10^{1,01}$, patient 32 of $10^{1,23}$ and patient 42 of $10^{1,34}$.

In the V+L+P+ group 16 patients (67%) had a lower expression compared to the patients with no micrometastases present (figure 3.22). The n-fold in the group of samples with a lower expression than the calibrator varied between –256.1 (patient 21, UEDKOJ) and -1.3 (patient 3, SARALE). The average expression of this group was 19.5 times weaker than the calibrator (SD 63). The expression of IFN-γ might be related to the micrometastatic state.

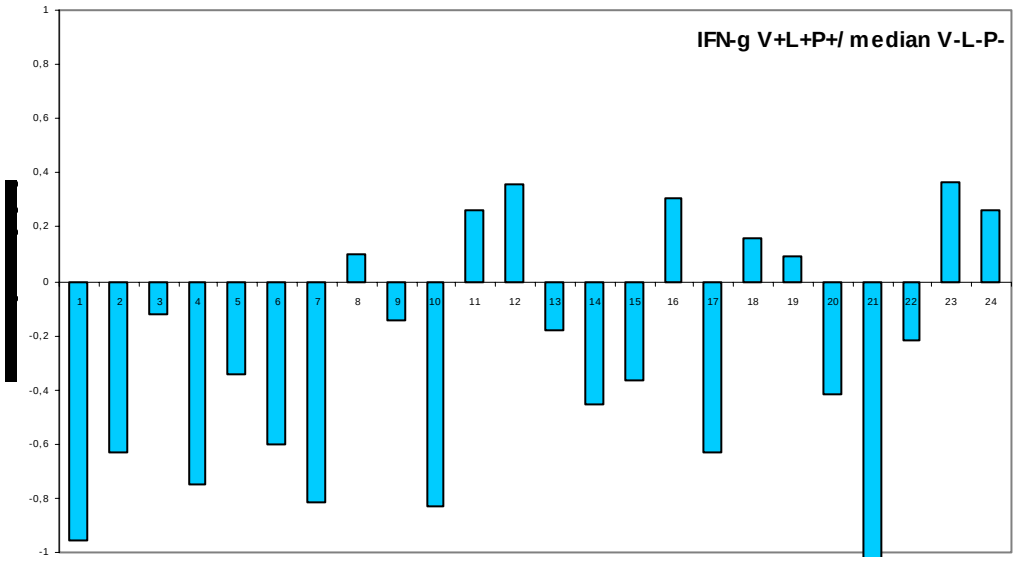



**Figure 3.22** Expression pattern of IFNg in V+L+P+ patients. Patient 21 has a value of $10^{-2,41}$.

***TGF-β*** The results of the suppressive cytokine TGF-β are illustrated in figure 3.23-3.24. Among the V-L-P- patients calibrated with the group with micrometastases present (figure 3.23) 56% (27/48) of the patients had a lower expression of TGF-β than patients with micrometastases. The expression in this group of 27 patients ranged from –4.8 (patient 27; AGURAL) to –1.0 (patient 15; HEIYVE). The expression of the 56% with a lower expression than the calibrator in the V-L-P- group was in average -2.1 (SD 1.1).

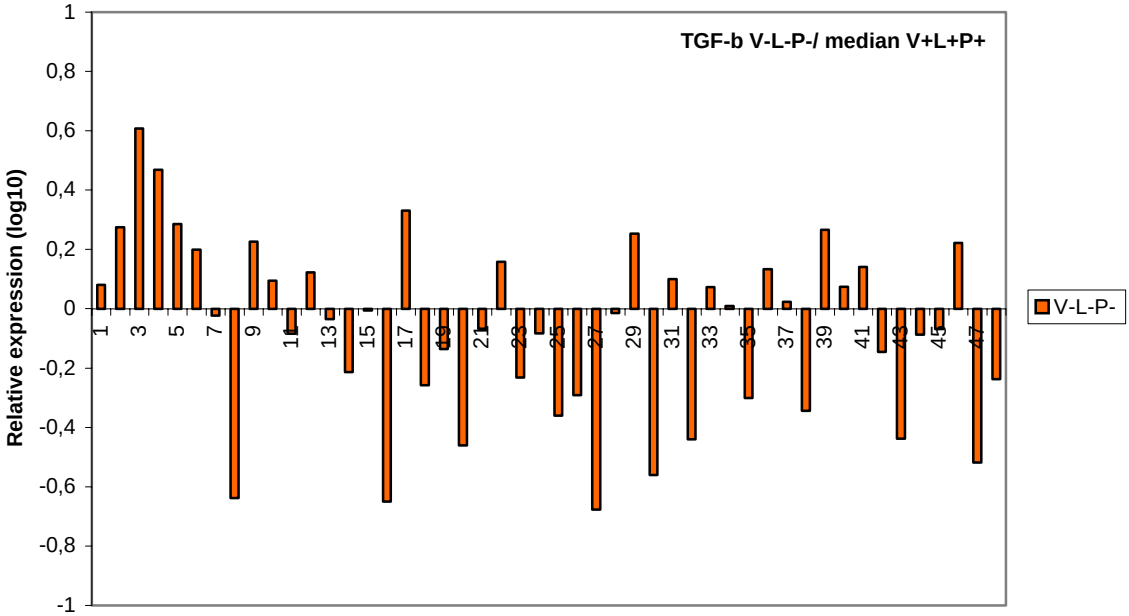



**Figure 3.23** Expression pattern of TGF-β in V-L-P- patients.

Figure 3.24 shows the graph with patients having some form(s) of micrometastase(s) present calibrated with the median of the patients without any micrometastases. Here 54% (13/24) of the patients had a higher expression of TGF-β than the V-L-P- calibrator. Among the 13 patients the expression varied between 1.1 times higher (patient 6 in the graph; DETFRE) and 5.8 times higher (patient 16 in the graph; BUUTHI) and the average expression was 1.9 times higher than in the V-L-P- group (SD 1.25).

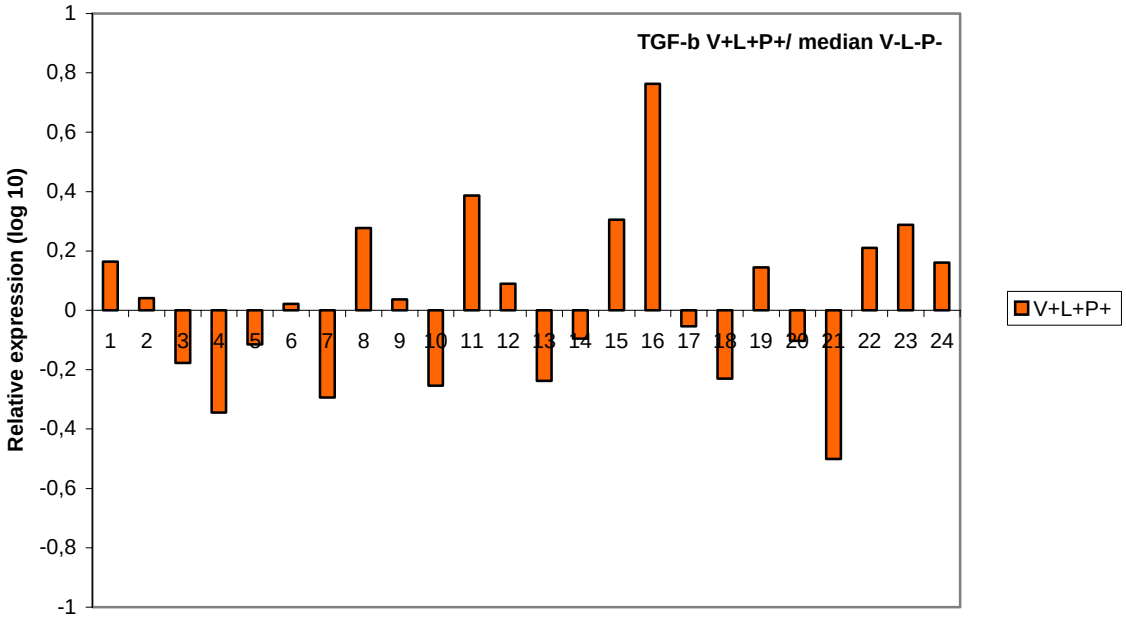



**Figure 3.24** Expression pattern of TGF-β in V+L+P+ patients.

# DISCUSSION

**The immune infiltrate present in colon cancers was estimated in a series of tumour specimens in correlation with the presence of micrometastases. RNA was extracted, reversely transcribed to cDNA and the expression determined with real time quantitative PCR. The preliminary results might indicate an association between the amount of CD8 expressing cells and the $T_H$ defence with micrometastases.**

The extractions of RNA generated a various number of degraded RNA depending on the technique used. The presence of degraded RNA is frequent in tissue extraction, in particular for tumour tissues. The quality of the samples of origin is often questioned. Among the quality affecting factors the time between the surgical removal of the tumour and the subsequent freezing of the tumour specimens seems decisive. The specimens included in this study were taken directly following the removal of the tumour and then instantly frozen in liquid nitrogen, a handling that should promote tissues of good quality. Additionally no thawing took place between the primary freezing and the homogenization preceding the extractions. The homogenization technique widely used and extensively validated by Qiagen did not seem to be implicated in the resulting RNA quality spectrum. This pointed out the fact that the extraction technique used affected the quality of the RNA. The extraction method of Applied Biosystems was performed in room temperature and the absence of protection of the tubes during the processing was perhaps a combination that made the samples more

susceptible to RNases. The comparison with the extraction method of Qiagen clearly demonstrated the superiority of this kit, both in matter of quality and amount of extracted RNA. The underlying causes of the discrepancy between the two kits are however difficult to analyse since the compositions of the different solutions included in the kits are not supplied. The comparison of the two extraction kits was enabled by the device for analysis of RNA, which permitted measurements of significant sensitivity.

The quality of the RNA was determined by analysing the ratio between 28S and 18S. In theory the ratio for non-degraded RNA is 2.7 but RNA with a ratio of 2 has generally been considered to be intact. However it is rare to see total RNA extracted from human tissues that actually exceed a ratio of 2. This may in part be linked to instability of the 28S rRNA structure relative to the 18S RNA. This means that even RNA with values well inferior to 2 may be of acceptable quality. To some degree the ratio may also vary with the tissue of origin.[14] To obtain the whole series with a ratio near 2 would have been a close to impossible project especially considering the cost and time as well as availability of tissue. Therefore the quality demand had to be inferior to this value. RNA with a ratio above 1.1-1.2 and with two clearly distinguishable ribosomal peaks were considered acceptable. It has been reported that partially degraded RNA exhibiting visible ribosomal bands demonstrate gene expression profiles similar to intact samples[15]. The quality differences between RNA extracted with ABI Prism™ and RNeasy® was shown as variability in quantity when tested with a quantitative PCR assay. When comparing RNA of different quality extracted with the RNeasy® kit these quantity differences were not visible. The explication for this was not obvious, but incited caution. Because of the need of homogeneous RNA for the quantitative PCR assays all RNA were re-extracted with the RNeasy® technology.

The low density array technique was appealing in many aspects. The technique was easy and not very time-consuming, and the used material in terms of cDNA (about 2 ng cDNA/PCR) and reagents was minimal. It was also a very reliable technique in that cDNA was amplified in all wells of all the cards used. The LDAs can be ordered in different configurations and with different genes. For this study the LDAs included 48 genes with room for 8 cDNAs per array. One replicate per sample was analysed. This choice (recommended by Applied Biosystems) was retrospectively supported by the homogeneity of the two samples analysed in two replicates.

The quality of the profiles obtained from the controls, i.e. the T lymphocytes and the tumoral lineages, was as anticipated. The curves of the populations of T lymphocytes were compatible with the cellular state of activation. The profiles of the T lymphocytes and the tumoral lineages were very dissimilar.

Understanding gene expression patterns in colon cancer disease will probably lead to the identification of genes relevant to patient prognosis. Real-time PCR with low density arrays provides the simultaneous measurement of gene expression in many different samples. To correctly interpret the results it is important to have a valid normalisation factor. This normalisation factor should correct the amount of starting material and differences in efficiency between different wells. Housekeeping genes have for long been used as normalisation factors. However housekeeping gene expression has been reported to vary, as could be seen in the amplification curves for 18S and GAPDH in this study. A normalisation factor based on several control genes is therefore preferable. Housekeeping genes can be rather constant in given cell types or under certain conditions, but can vary in different tissue types. This confers a problem because the tissues analysed naturally contain different cell

types in different amounts. Which housekeeping genes to choose is therefore not a trivial decision. The obtained results in this study were, as a first attempt, only normalised to one internal control. These observations should be verified by using multiple internal controls before proceeding to further LDA analysis or other techniques. Geometric averaging of multiple control genes as described by Vandesompele et al should result in a more accurate normalisation[16]. The correlation curve of 18S and GAPDH revealed two discording samples, which were removed from the analysis.

In PCR assays the expression level of the genes of interest can be determined either with absolute quantification or with relative quantification. In this study the relative quantification was chosen since the exact copy number was of little interest. The relative quantification could in turn be determined by different approaches, either with the comparative Ct method or with the standard curve method. The Ct method was advised by Applied Biosystems[17] and therefore chosen. For the $2^{-\Delta\Delta Ct}$ method to be valid the amplification efficiency of the target and the reference must be approximately equal. The amplification efficiency has been controlled on a number of TaqMan® assays by Applied Biosystems. All of the primers and TaqMan® probes have not been tested though, inciting a complementary study of a dilution series of one cDNA for the 48 genes.

The analysis of the results is still in progress as this report was written. The preliminary results could point out an association between the amount of CD8 expressing cells, the defence orientation ($T_H1$ or $T_H2$) and micrometastases. The amount of CD4 seemed unrelated to the micrometastatic state.

The CD8α mRNA is probably not a ubiquitously expressed gene in T lymphocytes. In fact the expression level of CD8α evaluated with cytometry can vary according to the state of activation. The expression can, in particular, be diminuated in presence of IL-4 [18]. CD8, although normally being a heterodimer, can be momentarily present as CD8α homodimers on CD8 T lymphocytes during the transition phase towards memory cells [19]. Despite these restrictions the tendency seen in the results of CD8 agree with the cytometry data earlier obtained in INSERM U255. The results for the role of CD8 are also consistent with earlier published data. The presence of CD8 in colon cancer has been shown to be strongly associated and highly predictive of MSI, microsatellite instability. This genetic condition is caused by defects of the mismatch repair system, which is involved in repairing DNA errors that arise during DNA replication. MSI appears to be related to better prognosis. It has not been demonstrated if the prognostic role of CD8 is a cause or a consequence of MSI.

The fact that CD4 seemed unrelated and TBX-21 and IFN-γ related to the micrometastatic state indicates that the importance concerning the T helper cells is presumably more dependent on the specific subset than the actual amount of T helper cells. A defence orientation is directed by cytokines but also the expression of transcription factors. IL-18 is a cytokine capable of promoting T cell IFN-γ production and facilitating $T_H1$ cell polarisation. IFN-γ secreted by $T_H1$ cells can inhibit the responsiveness by $T_H2$ cell. The results for IL-18 are not analysed yet.

TGF-β is a very strong immunosuppressor. It affects the activity of B lymphocytes, T lymphocytes and dendritic cells and has been reported to be locally produced in several cancers[20]. The hypothesis that high expression of TGF-β is correlated to presence of micrometastases could not be supported in this study. However it can be noted that the V-L-P-

group presents a weaker expression of TGF-β transcripts; the presence of TGF-β is deleterious for a $T_H1$ response.

Once all the results analysed with the $2^{-\Delta\Delta Ct}$ method, the results for the ensemble of the genes will be examined by clustering the different genes belonging to the same functional category. If several genes in one group point in the same direction this strengthens the hypothesis that this specific group is important in the development of micrometastases. The observations will be confirmed and extended with future investigations on Tissue Microarrays. This verification step will be a validation on proteins. The immunohistological technique of Tissue Microarrays will evaluate not only the proteins but also the cellular populations that express these proteins and their localisation in the tumour.

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# APPENDICES

## 6.1 Appendix I

### The genes included in the LDAs.

| Gene name | Function |
|---|---|
| *18S* | housekeeping gene |
| *GAPDH* | housekeeping gene |
| *CD66* | human tumour suppressor |
| *ACE* | tumour marker protein |
| *CD8* | T lymphocyte subtype |
| *CD4* | T lymphocyte subtype |
| *CD62L* | activation |
| *LAT* | activation |
| *OX-40* | activation/memory |
| *OX-40L* | activation |
| *ICOS* | activation/memory |
| *CXCL10* | $T_H1$ |
| *CXCL9* | $T_H1$ |
| *CXCR3* | $T_H1$ |
| *IFN-γ* | $T_H1$ |
| *IL-18Ra* | $T_H1$ |
| *IL-18Rb* | $T_H1$ |
| *IRF1* | $T_H1$ |
| *STAT-1* | $T_H1$ |
| *T-BET* | $T_H1$ |
| *IL-18* | $T_H1/T_H2$ |
| *GATA-3* | $T_H2$ |
| *IL-10* | anergy |
| *B7H1* | anergy/apoptosis |
| *B7H3* | anergy |
| *CD3Z* | anergy |
| *PD-1* | anergy |
| *TGF-b1* | anergy |
| *VEGF* | anergy |
| *granulysin* | cytotoxicity |
| *granzyme B* | cytotoxicity |
| *IRAK4* | inflammation |
| *COX-2* | inflammation |
| *TNF-α* | inflammation |
| *IL-8* | inflammation |
| *TNFRSF10A* | inflammation |
| *CD97* | tumoral escape |
| *survivin* | tumoral escape |
| *thrombospondin* | tumoral escape |
| *DCR3* | tumoral escape |
| *B7-DC/PDL2* | tumoral escape |
| *MMP7* | tumoral escape |
| *RCAS1* | tumoral escape |
| *CD32b (IRTA2)* | Other category |
| *INDO* | Other category |
| *LILRB3* | Other category |
| *ART1* | Other category |

## 6.2 Appendix II

**The classification criteria used for the patients.**

**T: Tumour**

| | |
|---|---|
| Tis | Intraepithelial tumour |
| T1 | Tumour invading the submucosa |
| T2 | Tumour invading the muscularis propria |
| T3 | Tumour invading the serosa |
| T4 | Tumour surpassing the serosa and/or invading adjacent organs |

**N: Lymph nodes**

| | |
|---|---|
| N0 | No metastased lymph nodes |
| Nx | Lymph nodes not evaluated or less than 12 lymph nodes examined |
| N1 | 1 to 3 lymph nodes with metastases |
| N2 | more than 3 lymph nodes invaded |

**M: Metastases**

| | |
|---|---|
| M1 | Distant metastases present |
| M0 | No metastases |

**Stages UICC**

| | |
|---|---|
| I | T1-T2, N0, M0 |
| II | T3-T4, N0, M0 |
| III | T1-4, N1-2, M0 |
| IV | T1-4, N1-2, M1 |

**Stages Dukes**

| | |
|---|---|
| A | T1-T2, N0, M0 |
| B | T3, N0, M0 |
| C | T1-4, N1-2, M0 |
| D | T1-4, N1-2, M1 |

**Micrometastases**

| | |
|---|---|
| V+ | Micrometastases present in blood vessels |
| V- | No micrometastases in blood vessels |
| L+ | Micrometastases present in lymphatic vessels |
| L- | No micrometastases in lymphatic vessels |
| P+ | Micrometastases present in perineural structures |
| P- | No micrometastases in perineural structures |

**Relapse**

| | |
|---|---|
| R- | No relapse |
| R+ | Relapse |

**Survival of the patient**

| | |
|---|---|
| V | Patient alive |
| M | Patient deceased |

## 6.3 Appendix III

**The samples included in the LDAs.**

| Patients | Type of tissue | Classification |
|---|---|---|
| LIMJUL | colon tumour tissue | T3 N0 M0 B2 V- L- P- R- V |
| ROGHEN | colon tumour tissue | T3 N2 Mx X V- L- P- R- V |
| SCAJAN | colon tumour tissue | T3 N1 M0 C1 V- L- P- R- M |
| THOJEA | colon tumour tissue | T4 N0 M0 B2 V- L- P- R+ M |
| MARJPI | colon tumour tissue | T3 N1 M0 C1 V- L- P- R- V |
| MATISA | colon tumour tissue | T3 N1 M1 D V- L- P- R+ V |
| MONREN | colon tumour tissue | T3 N0 M1 D V- L- P- R+ M |
| MORJUL | colon tumour tissue | T2 N0 M0 B1 V- L- P- R- V |
| MULWIL | colon tumour tissue | T4 N0 M0 B2 V- L- P- R+ M |
| BOUGIL | colon tumour tissue | T3 N0 M0 B2 V- L- P- R- V |
| BOUMAR | colon tumour tissue | T3 N0 M0 B2 V- L- P- R- V |
| BOUROB | colon tumour tissue | T3 N0 M0 B2 V- L- P- R- V |
| BRIFRA | colon tumour tissue | T2 N0 M0 B1 V- L- P- R- V |
| BURMAR | colon tumour tissue | T3 N0 M0 B2 V- L- P- R- V |
| HEIYVE | colon tumour tissue | T3 N1 M0 C1 V- L- P- R- M |
| HUBTHO | colon tumour tissue | T3 N1 M0 C1 V- L- P- R+ M |
| JULPIE | colon tumour tissue | T3 N1 M0 C2 V- L- P- R- M |
| KIEBER | colon tumour tissue | T3 N1 M1 D V- L- P- R- V |
| LEGCLA | colon tumour tissue | T3 N1 M1 D V- L- P- R+ M |
| LETRAY | colon tumour tissue | T3 N0 M0 B2 V- L- P- R+ M |
| LISGER | colon tumour tissue | T4 N0 M1 D V- L- P- R+ M |
| MARIRE | colon tumour tissue | T3 N1 M0 C1 V- L- P- R+ V |
| DOMMAU | colon tumour tissue | T1 N0 M0 A V- L- P- R- M |
| DRULIL | colon tumour tissue | T3 N0 M0 B2 V- L- P- R- V |
| FOUMAR | colon tumour tissue | T3 N1 M0 C1 V- L- P- R- V |
| ABDODE | colon tumour tissue | T3 N0 M1 D V- L- P- R+ V |
| AGURAL | colon tumour tissue | T3 N1 M0 C1 V- L- P- R+ M |
| AMMAOM | colon tumour tissue | T3 N1 M0 C1 V- L- P- R- V |
| AUDYVE | colon tumour tissue | T3 N1 M1 D V- L- P- R+ M |
| BEHCHR | colon tumour tissue | T3 N1 M1 D V- L- P- R+ M |
| BENDAV | colon tumour tissue | T2 N0 M0 B1 V- L- P- R- V |
| BIDCOL | colon tumour tissue | T2 N0 M0 B1 V- L- P- R+ V |
| CARGIL | colon tumour tissue | T3 N1 M0 C2 V- L- P- R+ M |
| CARSUZ | colon tumour tissue | T3 N0 M0 B2 V- L- P- R- V |
| CHEYVE | colon tumour tissue | T3 N1 M1 D V- L- P- R+ M |
| DAIDAN | colon tumour tissue | T3 N1 M0 C2 V- L- P- R- V |
| DANLUC | colon tumour tissue | T3 N0 M0 B1 V- L- P- R- V |
| DEBTHE | colon tumour tissue | T1 N0 M0 A V- L- P- R- V |
| DEFCYR | colon tumour tissue | T3 N0 M0 B2 V- L- P- R- V |
| DELJAN | colon tumour tissue | T3 N0 M0 B2 V- L- P- R- M |
| DEMCHR | colon tumour tissue | T3 N1 M0 C2 V- L- P- R- V |
| NASEST | colon tumour tissue | T3 N1 M0 C1 V- L- P- R- V |
| NDONDO | colon tumour tissue | T2 N0 M1 D V- L- P- R+ M |
| OL1AME | colon tumour tissue | T3 X M1 D V- L- P- R+ V |
| OLIAME | colon tumour tissue | T3 X M1 D V- L- P- R+ V |
| PIEROB | colon tumour tissue | T3 N1 M0 C1 V- L- P- R- M |
| PIRCHR | colon tumour tissue | T3 N1 M1 D V- L- P- R+ M |
| PORGEO | colon tumour tissue | T3 N1 M1 D V- L- P- R+ M |
| BEGBAY | colon tumour tissue | T4 N0 M1 D V- L- P- R+ V |
| LESMAR | colon tumour tissue | T4 N0 M0 B2 V- L- P- R+ M |
| BOUPAT | colon tumour tissue | T3 N1 M1 D V- L- P+ R- M |
| BEAMIC | colon tumour tissue | T4 N1 M1 D V- L+ P- R+ V |
| SARALE | colon tumour tissue | T3 N1 M0 C1 V- L+ P- R- V |

| | | |
|---|---|---|
| SIGGEN | colon tumour tissue | T3 N1 M1 D V- L+ P- R+ M |
| CHEVAZ | colon tumour tissue | T3 N1 M1 D V- L+ P- R+ M |
| COSMAR | colon tumour tissue | T3 N1 M1 D V- L+ P- R+ M |
| DETFRE | colon tumour tissue | T3 N1 M1 D V- L+ P- R+ M |
| MOINIC | colon tumour tissue | T4 N0 M1 D V- L+ P+ R+ M |
| PLOHAR | colon tumour tissue | T2 N1 M0 C1 V- L+ P+ R- M |
| DUCPIE | colon tumour tissue | T3 N1 M0 C2 V+ L- P- R- V |
| DULPIE | colon tumour tissue | T3 N0 M1 D V+ L- P- R+ M |
| HARPIE | colon tumour tissue | T3 N1 M0 C1 V+ L- P- R- V |
| DESAMA | colon tumour tissue | T3 N1 M0 C1 V+ L- P- R- V |
| VILMAR | colon tumour tissue | T3 N1 M0 C1 V+ L- P- R- V |
| ROMDEN | colon tumour tissue | T3 N1 M1 D V+ L- P+ R+ M |
| SAIGER | colon tumour tissue | T3 N1 M0 C1 V+ L- P+ R+ V |
| BUUTHI | colon tumour tissue | T3 N0 M0 B2 V+ L- P+ R- V |
| BOUHEN | colon tumour tissue | T3 N1 M0 C2 V+ L+ P- R- V |
| BILDOM | colon tumour tissue | T3 N0 M0 B2 V+ L+ P- R- V |
| CARPIE | colon tumour tissue | T3 N1 M1 D V+ L+ P- R+ M |
| CHAANN | colon tumour tissue | T3 N0 M0 B2 V+ L+ P- R- M |
| UEDKOJ | colon tumour tissue | T2 N1 M0 C2 V+ L+ P+ R+ V |
| MORALA | colon tumour tissue | T3 N1 M1 D V+ L+ P+ R+ V |
| HALKIR | colon tumour tissue | T3 N1 M1 D V+ L+ P+ R+ V |
| DAVDAN | colon tumour tissue | T4 N1 M1 D V+ L+ P+ R+ M |
| MONCHA | colon tumour tissue | T3 N0 M0 B2 x x x R- V |
| DUBAME | colon tumour tissue | T4 N1 M1 D x x x R+ V |
| MIX CT#1 | colon tumour tissue | |
| MIX CT#2 | colon tumour tissue | |
| SAIGER | normal colon tissue | |
| OLIAME | normal colon tissue | |
| DESAMA | normal colon tissue | |
| BIDCOL | normal colon tissue | |
| MORJUL | normal colon tissue | |
| HEUAND | normal colon tissue | |
| MIX CN | normal colon tissue | |
| ASSAYAG | liver tumour tissue | |
| SAIGER | liver tumour tissue | |
| KIEBER | liver tumour tissue | |
| MAZCLE | liver tumour tissue | |
| VAUTHIER | liver tumour tissue | |
| HEIJEA | liver tumour tissue | |
| CRIPAS | liver tumour tissue | |
| MIX FN | normal liver tissue | |
| SW480 | colon tumour lineage (ADCC) | |
| HT29 | colon tumour lineage (ADCC) | |
| SW620 | colon tumour lineage (ADCC) | |
| CaCo2 | colon tumour lineage (ADCC) | |
| LS174T | colon tumour lineage (ADCC) | |
| LOVO | colon tumour lineage (ADCC) | |
| LT 3/28/TGF 09/04/02 | purified T lymphocytes | |
| LT NS 17/07/03 | purified T lymphocytes | |
| LT 3/28/IL-2 17/07/03 | purified T lymphocytes | |
| LT 3/28/IL-10 17/07/03 | purified T lymphocytes | |
| LT NS 22/10/03 | purified T lymphocytes | |
| LT b-CD3 22/10/03 | purified T lymphocytes | |
| LT b-B7H1 22/10/03 | purified T lymphocytes | |
| LT 3/28/IL-7 08/04/03 | purified T lymphocytes | |
| LT 3/28/IL-15 19/06/03 | purified T lymphocytes | |
| LT 3/28 09/04/03 | purified T lymphocytes | |

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