

**Charles University in Prague
Faculty of Medicine in Hradec Králové**

PHD DISSERTATION THESIS

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MBBS MRCP (UK)**

2015

**Charles University in Prague
Faculty of Medicine in Hradec Králové**

Doctoral degree programme

Internal Diseases

Title

Clinical use of neopterin, a laboratory biomarker of immune activation, in the assessment of prognosis, monitoring response to therapy and complications in cancer patients

Titul

Klinické použití neopterinu, laboratorního biomarkeru imunitní aktivity, v odhadu prognózy, monitorování odpovědi na léčbu a komplikací u pacientů s nádorovým onemocněním

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Annex No. 2

Author's Declaration

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Hradec Králové, 2015

Signature of the author

Acknowledgements

I wish to express my deepest gratitude to my supervisor Professor Bohuslav Melichar who continues to give me endless guidance and encouragement. He has been the source of inspiration that kept me going. Without his ideas and constructive criticisms in addition to his tireless efforts, this work would not have been possible.

I would also like to thank Prof. Jan Bures for being patient and supportive during the ups and downs of my work.

Prof. Jiri Petera has been a pillar of support and was always there to listen to all my ideas. He has been a great mentor during my clinical work for the studies. Professor Zadak needs a special mention as his guidance and help were instrumental in my pursuit of an academic degree. Doc. Solichova was fabulous during my attachment in her laboratory to gain insight into the laboratory procedure and data collection.

Without her the study would not have been possible

A special thanks to my colleagues, Martin Majirsky, Pavel Vesely, Docent Svoboda and Prof. Stanislav Filip at the department of Oncology and Radiotherapy at the Faculty hospital were a tremendous help during my work at the department. My friends Dr Shriharsh Pilatadka, Dr. Sujith Sumkumar, Dr.Pratima Singh, and Dr.Poorva Deshapande cheered for me to carry on and made the study enjoyable. I also express my gratitude to my extended family in Yavatmal who always stood by me and supported my decisions.

Finally to my wife Vidhi, thank you for all your love and care during months of long hours it took to compile this thesis. You have been amazing.

This work is dedicated to my father
Mr. Vipinchandra Dayashankar Trivedi

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1. 1 Souhrn

Název práce

Klinické použití neopterinu, laboratorního biomarkeru imunitní aktivity, v určení prognózy, monitorování odpovědi na léčbu a komplikací u pacientů s nádorovým onemocněním

Uvod

Neopterin je biomarker imunitní aktivity, syntetizovaný z GTP v reakci katalyzované enzymem GCH-1. Hladiny neopterinu odrážejí reakci organismu na zánětlivé stavy, jako jsou infekce, poranění, chronických onemocnění a rakovina. Hladina kolísá i v průběhu všech modalit protinádorové terapie, která ovlivňuje činnost imunitního systému. Vysoká hladina neopterinu je spojena se špatnou prognózou u nádorových onemocnění.

Cíl

Ověření klinického použití neopterinu, ve stanovení prognózy, monitorování odpovědi na léčbu a komplikací u pacientů s rakovinou.

Metodologie

Ve dvou částech studie, sériové neopterinu v moči byly měřeny ve dvou různých kohortách pacientů, kteří podstoupili protinádorovou terapii. V první části, byly analyzovány vzorky od 45 pacientů s diagnózou metastazujícího kolorektálního karcinomu, kteří byli léčeni chemoterapií v kombinaci s cetuximabem. Ve druhé části byly analyzovány vzorky 10 pacientek B s diagnózou gynekologické malignity, většinou karcinomu děložního hrdla, podstupujících chemoradioterapii.

Výsledky

U nemocných s metastatickým kolorektálním karcinomem byly vyšší hladiny neopterinu spojeny se špatnou prognózou. Hladina neopterinu korelovala s koncentrací hemoglobinu, počtem bílých krvinek a koncentrací CEA. U nemocných s gynekologickými malignitami byly výchozí hladiny neopterinu vyšší. Byl pozorován vzestup neopterinu v souvislosti s komplikacemi.

Závěr

Tato data prokazují potenciální klinické využití neopterinu, v prognóze, monitorování odpovědi na léčbu a komplikací u pacientů s rakovinou. Další studie na větších kohortách pacientů jsou potřeba k zavedení stanovení neopterinu do širší klinické praxe.

1. 2 Summary

Title

Clinical use of neopterin, a laboratory biomarker of immune activation, in prognosis, monitoring response to therapy and complications in cancer patients

Introduction

Neopterin is a biomarker of immune activation and is synthesized from GTP in a reaction catalyzed by enzyme GCH-1. Neopterin levels reflect the body's response to inflammatory conditions such as infections, injuries, chronic diseases, and cancer. Its levels also fluctuate with anticancer therapies that demonstrate immune activity. Remarkably neopterin has also been found to be a marker of poor prognosis in cancer.

Aim

To investigate clinical use of neopterin, a biomarker of immune response, in the assessment of prognosis, monitoring response to therapy, and complications in cancer patients.

Methodology

In a two-part study, serial urinary neopterin were measured in two different cohorts of patients who underwent anticancer therapy. In part one, samples from 45 patients with diagnosis of metastatic colorectal cancer who were being treated with chemotherapy + cetuximab were analyzed. In part two, samples from 10 patients with diagnosis of gynecological malignancy, mostly cervical cancer, and undergoing chemoradiotherapy were analyzed.

Results

In patients with metastatic colorectal carcinoma, higher neopterin levels were associated with poor prognosis. In this cohort, neopterin levels showed correlation to hemoglobin levels, white cell count and CEA. In patients with gynecological cancer, pretreatment neopterin levels were generally higher. No association of therapy-associated changes in neopterin levels were observed, however, we were able to demonstrate that the rises in neopterin were related with complications.

Conclusion

With our data we have been able to demonstrate the potential clinical uses of neopterin in prognosis, monitoring response to therapy and complications in cancer patients. However, much larger studies with different tumor types could be performed to corroborate and refine the methodology to put neopterin in clinical practice.

Abbreviations used

5-FU – 5 Fluorouracil
(ADCC)- Antibody dependent cell-mediated cytotoxicity
AIDS- acquired immunodeficiency syndrome
BMI- Body Mass Index
BH4- Tetrahydrobiopterin
Ca⁺⁺- Calcium Ion
CD- Cluster of differentiation
CDC complements dependent cytotoxicity
DC dendritic cells (DCs)
EGFR- Epidermal Growth Factor Receptor
GTP – Guanosine triphosphate
GCH 1 - GTP-cyclohydrolase I
G-CSF – Granulocyte colony stimulating factor
GM-CSF – Granulocyte monocyte colony stimulating factor
HIV – Human immunodeficiency Virus
HOCL- Hypochlorous acid
HSP – Heat shock Protein
iNOS - inducible nitric oxide synthase
IDO - indoleamine (2,3)-dioxygenase
IFN- alpha – Interferon Alpha
IFN-gamma – Interferon Gamma
IL-2 – Interleukin 2
IL- 6 - Interleukin 6
IL-10 – Interleukin 10
IL-12 - Interleukin 12,
Kyn/tryp - kynurenine to tryptophan ratio
KRAS - Kirsten rat sarcoma viral oncogene homolog
LPS - lipopolysaccharide
mAb- monoclonal antibody
NK - natural killer
NF-κB - nuclear factor-κB
OSA – Obstructive sleep apnea
PTPS 6 - Pyruvoyltetrahydropterin synthase
rIFN- gamma recombinant IFN-gamma
RT- Radiotherapy
TNF-alpha Tumour necrosis factor Alpha
Tryp - Tryptophan
TAM - Tumour-associated macrophages
Zn - Zinc

2. Introduction

Malignant tumors elicit host response that involves both the adaptive and innate immune systems [Melichar et al 2006a]. Body's defense mechanism is designed to eliminate aberrant cells. However, the development of cancers demonstrates that these responses are not always sufficient to preclude malignancy. It is now evident that cancerous cells manage to escape immune recognition and elimination [Dranoff 2004] by evolving certain strategies to escape from immune surveillance. This quality of cancerous cell is known to be an important aspect in early pathogenesis of malignant disease [Boon and Der Bruggen 1996]. In addition, the crosstalk between normal and neoplastic cells is a factor that influences various stages of carcinogenesis [Hanahan & Weinberg 2000]. An important variable that might prove decisive in molding the host reaction is the mixture of cytokines that is produced in the tumor microenvironment [Dranoff & Mulligan 1995]. Thus the immune response to cancer cells and the escape mechanism of latter could be studied by studying the cytokines, the cellular components and the products of host response in tumor microenvironment and also in various visceral fluids [Melichar et al 1998].

IFN-gamma is one such cytokine produced by the T-lymphocytes and natural killer cells. Interestingly, IFN-gamma has been found to play an important role in regulating tumor growth [Ikeda et al 2002] in different ways. For example IFN- γ may play two distinct roles in expressing the antitumor efficacy of IL-12: one to support the T-cell acceptability of tumor masses, and the other to mediate the antitumor effects of migrated T cells [Ogawa et al 1998].

Upon stimulation by IFN-gamma the monocyte/macrophage and dendritic cells show enhanced production of pteridines such as neopterin [Murr et al 2002, Weirleitner et al 2002]. Other cell types like, endothelial cells [Andert et al 1992], B-lymphocytes [Hoffmann et al 1992], and kidney cells [Mountabarrick et al 1994] also produce neopterin. However, neopterin output from monocyte/macrophages has been found to be higher several folds to several orders of magnitude [Melichar et al 2006a]. In addition to the activation of immune system high neopterin production is also associated with increased production of reactive oxygen species and with low serum concentrations of antioxidants like α -tocopherol. Hence, it can also be regarded as a marker of reactive oxygen species formed by the activated cellular immune system

[Murr et al 2002]. Therefore, by measuring neopterin not only the extent of cellular immune activation, but also the extent of oxidative stress can be estimated [Murr et al 2002, Hoffmann et al 2003].

Neopterin was first isolated from human urine by Sakurai and Goto in 1960's [Sakurai and Goto 1967]. It was found to be one of the molecules responsible for the fluorescence of urine in cancer patients [Wachter et al 1989]. Fuchs et al. highlighted neopterin's association to immune system in in the early 1980s when they proposed neopterin to be a marker of immune activation [Fuchs et al 1984].

Chemistry and synthesis

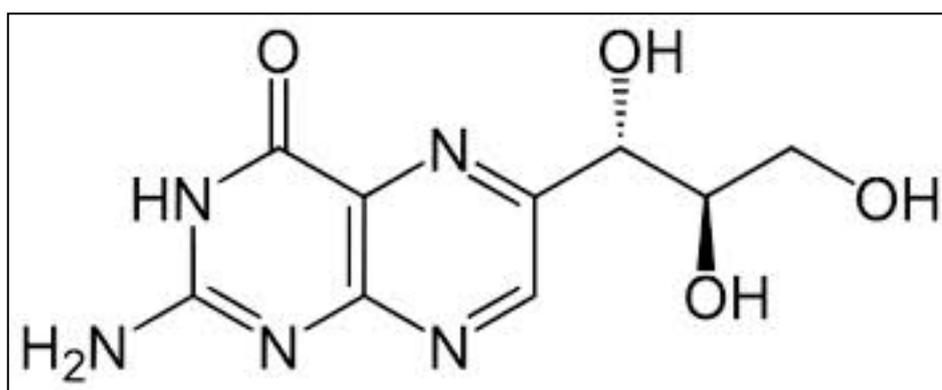


FIGURE 1. Neopterin: 2-amino -4-oxo-6 -(derythro-1', 2', 3'-trihydroxypropyl)-pteridine [1]

Chemically, neopterin is 2-amino -4-oxo-6 -(derythro-1', 2', 3'-trihydroxypropyl)-pteridine, which is an unconjugated pteridine. It is synthesized from Guanosine Triphosphate (GTP) by the action of GTP cyclohydrolase 1 (GCH-1) [Melichar et al 2006a].

In a reaction catalyzed by GCH-1, GTP is converted to 7, 8-dihydroneopterintriphosphate, which is the first step towards formation of neopterin [Becker et al 2013].

Next, 7, 8- dihydroneopterintriphosphate, is metabolized in two different ways depending on the location. Pyruvoyltetrahydropterin synthase (PTPS) is the enzyme responsible for conversion of 7, 8- dihydroneopterintriphosphate to 5,6,7,8 – Tetrahydrobiopterin (BH4). Most of the somatic cells produce BH4 by this route; however, the story is different in macrophages.

IFN gamma can stimulate the production of GCH-1 in various cell types. However, in macrophages the degree of induction of GCH-1 activity is significantly higher than the constitutively present 6-pyruvoyl tetrahydropterin synthase (PTPS) activity. [Murr et al 2002]

Thus in macrophages and dendritic cells the IFN gamma induced GCH-1 production leads to increased accumulation of 7, 8- dihydroneopterintriphosphate which acts as the substrate of neopterin production. In contrast, in other cells GCH-1 activity always remains lower than the PTPS synthase activity leading to BH4 synthesis without the accumulation of large amounts of neopterin derivatives [Werner et al 1990].

Though no clear reason for the low expression PTPS synthase in human macrophages has been identified, exon skipping has been demonstrated to be a mechanism responsible [Leitner et al 2003]. Leitner et al demonstrated that PTPS transcripts in macrophages lack exon 3 resulting in premature stop codon. PTPS transcripts lacking exon 3 are also characteristic of dendritic cells, myeloid cell lines, B-lymphocytes, and T-lymphocytes, but monocytes/macrophages remain the most important cell population responsible for neopterin production. [Leitner et al 2003].

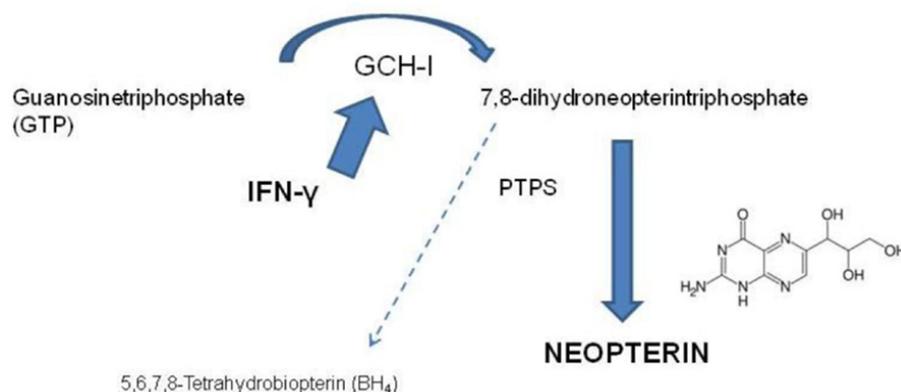


FIGURE 2. In various cells the Th1-type cytokine IFN-gamma (IFN- γ) induces the GTP-cyclohydrolase I (GCH I) to produce 7, 8-dihydroneopterintriphosphate. Due to a deficiency in 6 -pyruvoyltetrahydropterin synthase (PTPS) in human monocyte-derived macrophages and dendritic cells, the production of 5, 6, 7, 8-tetrahydrobiopterin is almost zero and neopterin is produced in high concentrations. [Becker et al 2013]

The next step toward the production of neopterin involves conversion of 7, 8-dihydroneopterintriphosphate to neopterin. This is carried out by the dephosphorylation of 7, 8- dihydroneopterintriphosphate and further oxidation. HOCL is a cytotoxic compound that has been recognized as the agent that can oxidize 7, 8-dihydroneopterintriphosphate to form neopterin [Widner et al 2000, Gieseg et al 2001].

7, 8-dihydroneopterin is the intermediate in the process of formation of neopterin. It has been found that the ratio of 7, 8-dihydroneopterin to neopterin is constant [Fuchs et al 1989], and therefore neopterin concentrations can be used to assess in vivo GTP cyclohydrolase I activity and consequently, activation of the macrophages [Melichar et al 2006a].

The macrophages produce neopterin out of the 7, 8-dihydroneopterintriphosphate intermediate at the expense of 5, 6, 7, 8-tetrahydrobiopterin (BH4). As a result the levels of this cofactor required for synthesis of several aromatic amino acid hydroxylases and nitric oxide synthases runs low in macrophages. Fuchs et al. have proposed this biochemical peculiarity of pteridines' biochemistry as a reason for hampered nitric oxide production in human monocyte-derived macrophages [Fuchs et al 1994a].

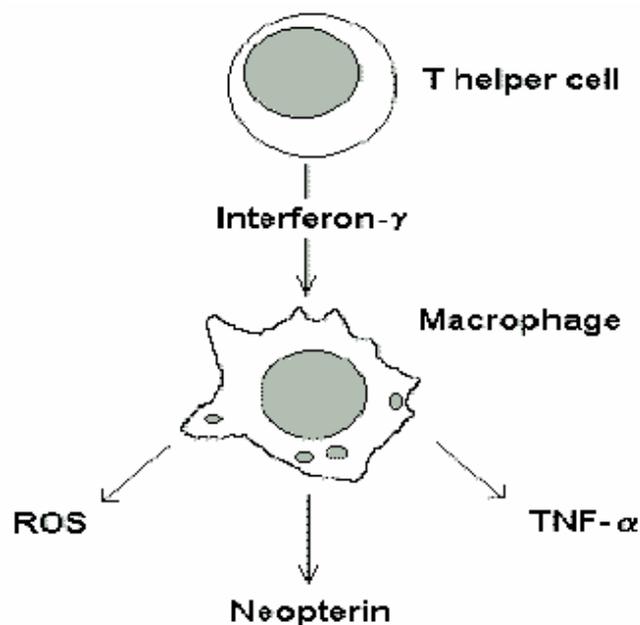


FIGURE 3 : IFN-Gamma induced production of ROS, Neopterin and TNF alpha [Murr et al 2001]

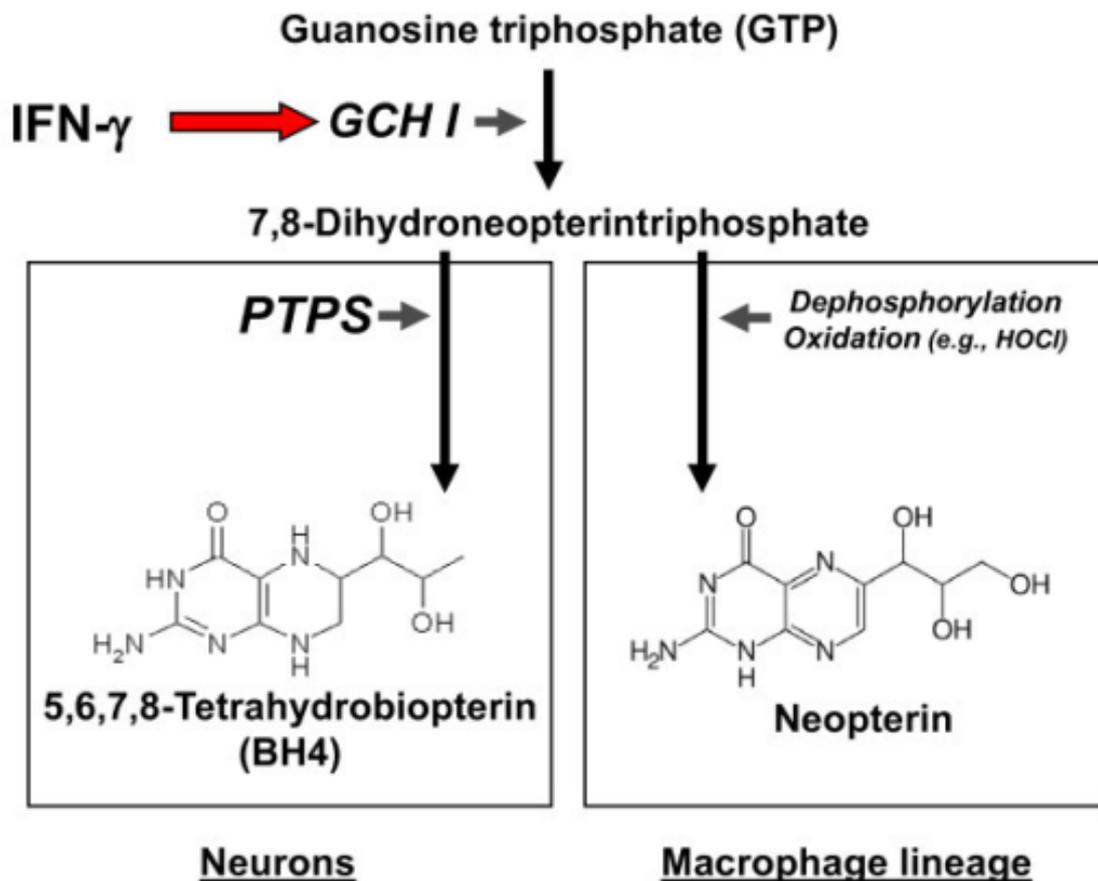


FIGURE 4: Induction of neopterin formation in brain cells. Proinflammatory cytokines like interferon- γ (IFN- γ) induce expression of GTP-cyclohydrolase I in various brain cells. As an intermediate product 7, 8-dihydroneopterin-triphosphate is produced which is further converted by pyruvoyl-tetrahydropterin synthase (PTPS) to form 5, 6, 7, 8-tetrahydrobiopterin (BH4), the cofactor of several aromatic amino acid monooxygenases that are involved in the production of tyrosine, L-DOPA, serotonin and nitric oxide. Different from neurons, monocytic cells possess only low constitutive activity of PTPS. Thus, 7, 8-dihydroneopterin-triphosphate does not undergo conversion to BH4, rather it is dephosphorylated and oxidized to neopterin in non-enzymatic reactions. [Hagberg 2010]

The above illustration shows the difference between pteridine metabolism by macrophages and other cell (e.g. neurons).

Elimination

Neopterin is eliminated by the kidneys and changes in the neopterin concentrations in serum are reflected by corresponding alterations of urine levels as long as renal function is normal [Fuchs et al 1994b]. A strong correlation between urinary and serum neopterin levels has been established [Fuchs et al 1988a] and in the absence of renal insufficiency both serum (or plasma) and urine may be used for the measurement of neopterin [Melichar et al 2006a].

Renal clearance of neopterin is similar to that of creatinine. Therefore, neopterin per creatinine ratios in urine are not influenced by renal impairment [Fuchs et al 1994b]. Unlike that, blood neopterin concentrations depend on renal function; reduced renal excretion causes accumulation of neopterin in blood and one may find extremely high values in serum or plasma in patients with uremia [Fuchs et al 1988b]. Thus, in patients with impaired renal function accumulation of neopterin may occur in the blood that is in addition to the enhanced formation of neopterin by immune system activation. Calculating the neopterin per creatinine ratio is suitable to at least partly account for the accumulation due to deterioration of renal function [Fuchs et al 1988b].

Measurements

Neopterin and 7, 8-dihydroneopterin have a small molecular mass (253 and 255 D), and are produced and released in a remarkably constant proportion with a ratio of aromatic neopterin to total (aromatic plus acid-oxidizable 7,8-dihydroneopterin) neopterin of 1:3 for urine and arterial blood and 1:2 for serum obtained from venous blood samples [Murr et al 2002].

Since dihydroforms of pteridines are labile, collection and storage of samples is critical and problematic for large scale clinical handling. In daily clinical routine, advantageously only the more stable neopterin is quantified [Murr et al 2002]. Neopterin is sensitive to direct sunlight and irradiation and therefore samples must be protected from light during transport and storage prior to measurement of neopterin [Laich et al 2002]. The fresh samples that are collected could be protected e.g. by enveloping in aluminum foil, alternatively dark tubes may be used for collecting samples. An immediate transfer into dark freezer would be a logical step before transit to the laboratory for measurements.

Neopterin levels can be determined in various bodily fluids such as serum, plasma, CSF, and urine. In serum, plasma and other protein-containing body fluids, e.g. cerebrospinal fluid, pancreatic juice or ascites, neopterin levels are preferably determined by immunoassays (ELISA or radioimmunoassay) [Mayersbach et al 1994]. For the determination of neopterin in urine samples high pressure liquid chromatography (HPLC) on reversed phase is usually applied [Schroecksnadel et al 2006]. Neopterin is detected by measurement of its natural fluorescence (excitation wavelength 353 nm, emission wavelength 438 nm), creatinine concentrations can be measured in parallel in the same chromatographic run by detection of its UV-absorption at 235 nm wavelength.

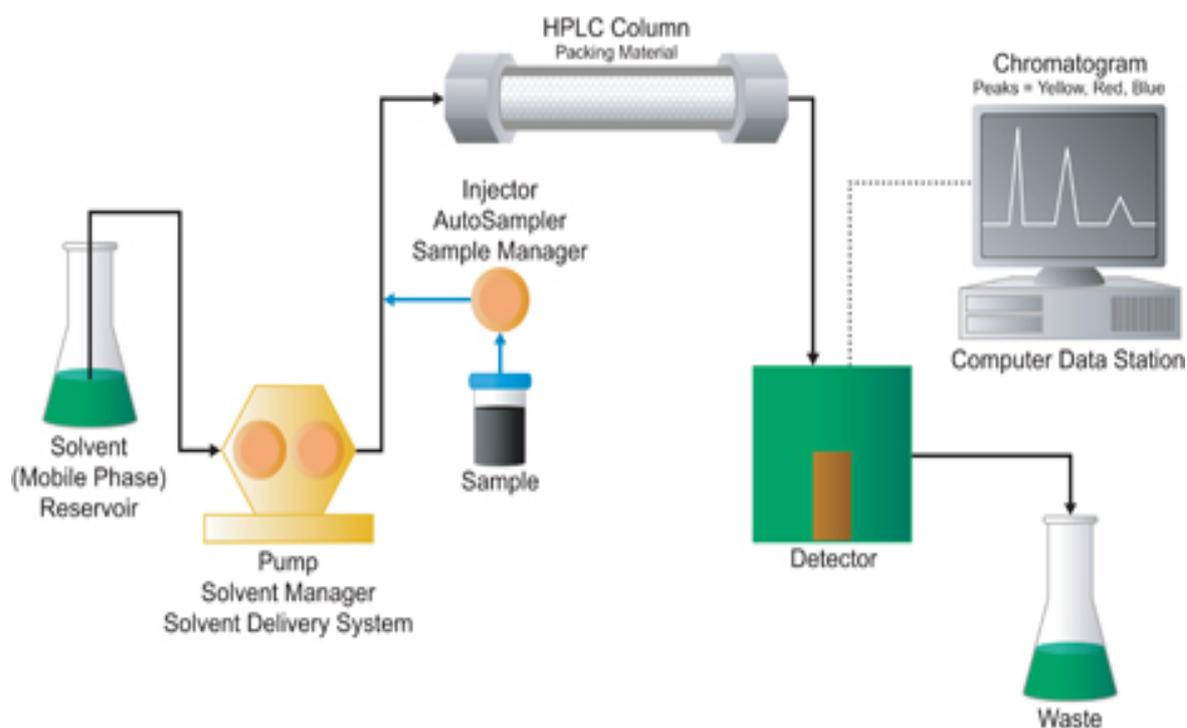


FIGURE 5. HPLCC apparatus and setup [Waters.com]

HPLC measurements of neopterin concentrations in serum or plasma are limited by the fact that protein precipitation will increase neopterin content of samples because of oxidation of its 7, 8-dihydroneopterin derivative [Werner et al 1987]. Thus, size-exclusion filter cartridges or precipitation can achieve preanalytical separation of protein with non-acidic reagents such as acetonitrile [Flavall et al 2008]. No significant difference in the neopterin concentrations in serum or plasma has been found.

Table 1: Neopterin concentrations (mean \pm S.D. and 97.5th percentiles) in urine of healthy individuals [neopterin.net]

Neopterin in urine ($\mu\text{mol/mol}$ creatinine):

Age	Male	97.5 th	Female	97.5th
19-25	123+30	195	128+33	208
26-35	101+33	182	124+33	209
36-45	109+28	176	240+39	239
46-55	105+36	197	147+32	229
56-65	119+39	218	156+35	249
>65	122+38	229	151+40	251

Normal values and upper limits of tolerance of urinary neopterin concentrations in healthy persons would change depending on age and sex, which is mainly due to variations of urinary creatinine concentrations [neopterin.net]. For example in a young person's neopterin concentrations detected in the first morning urine are about 1500 nmol/l, concentrations of creatinine about 12 mmol/l; thus, the normal value of healthy persons is calculated as about 125+5 $\mu\text{mol/mol}$ creatinine. These variations need to be taken into account when performing clinical studies.

3. Background

Neopterin in human pathology

Localized or systemic inflammation is a feature of a large proportion of human pathologies. Recent investigations have also found links of inflammation to the development of cancer [Hagemann et al 2007, Laird et al 2011]. In fact inflammation is now labeled as one of the hallmarks of cancer [Colotta et al 2009]. A direct association of inflammation to some cancer like esophageal cancer with barrett's oesophagus and inflammatory bowel disease with colorectal cancer is also well known.

A review points out that about 15% of the global cancer burden is attributable to infectious agents and inflammation is a major component of these chronic infections. Moreover, increased risk of malignancy is associated with the chronic inflammation caused by chemical and physical agents, and autoimmune and inflammatory reactions of uncertain aetiology [Balkwill and Mantovani 2001]. Chronic inflammation invariably leads to a chronic immune stimulation.

Hence, the study of cytokines and biomarkers to monitor the immune activity can provide an insight in the processes involved in cancer development and can lead to new concepts and strategies for its management. This activity of systemic inflammatory or immune response may be studied by measuring serum or plasma cytokine concentrations; however, cytokines are notorious for their marked fluctuations of systemic concentrations [Melichar 2013]. It could therefore be argued that the measurement of stable biomarkers that could reflect the immune activity can be a logical alternative.

Although in current practice biomarkers play an increasingly important role in the management of cancer patients, the utilization of biomarkers associated with host response to malignancy has been limited [Melichar 2013]. It is also worth noting that serial cytokine determinations for the longitudinal study of inflammatory phenomena and the measurement of routine markers of inflammation such as CRP and other acute phase reactants require venipuncture, which is not only uncomfortable for the patients but also has potential complications in addition to the administrative and cost implications. Hence, there is a place for a compound that would overcome these obstacles.

Neopterin and chronic immune stimulation

As described earlier, neopterin is produced from GTP by macrophages upon activation of GCH-1 by INF gamma, which is a cytokine produced by T-lymphocytes and natural killer cells. Because the production of IFN- gamma is enhanced by pro-inflammatory cytokines such as interleukin-1 or interleukin-6, systemic concentrations of neopterin accompany both systemic immune and inflammatory response [Wachter et al 1989].

The general state of immune activation in patients with advanced cancer, evidenced by increased neopterin production and increased expression of lymphocyte activation markers like CD69 or HLA-DR, may be a consequence of the failure of the host immune system to cope with the tumor [Melichar et al 2006a]. We know from studies that chronic immune stimulation in cancer patients is associated with alterations in leukocyte phenotype and function [Melichar et al 2006a]. In addition, 7, 8-dihydroneopterin has been shown to induce apoptosis in freshly isolated human T-lymphocytes in a concentration-dependent manner [Baier-Bitterlich et al 1995]. Thus chronic immune activation may lead to a qualitative and quantitative impairment of the host defense that would be responsible hampering adequate immune response.

The net result of these complex immune activities would again influence the levels of neopterin that can be easily observed by analyzing the urine of the patient.

Neopterin and prognosis in cancer patients

Neopterin has been of particular interest in oncology. There have been studies linking neopterin to diagnosis and prognosis of the underlying malignant disease.

It has been demonstrated that the sensitivity of the urine neopterin in diagnosis varies according to the type and stage of cancer [Bayram et al 2004]. Reports suggest that this sensitivity reaches almost 100% accuracy in cases of hematologic neoplastic condition (non-Hodgkin's lymphoma, chronic lymphoblastic leukemia) [Abate et al 1989]. On the other hand the data for different solid tumor was found to be variable. The frequency of increased neopterin in other cancers such as gynecologic cancers [Reibnegger et al 1986, Reibnegger et al 1987], lung cancers [Conrad et al 1987] and

cancers of colon ranges over 50% [Putzki et al 1987]. However, the sensitivity in breast cancer has been found to be much lower at around 20% [Wiegele et al 1984].

Studies in patients with hematological malignancies revealed that neopterin concentrations correlate with tumor stage of non-Hodgkin and Hodgkin lymphomas and might be considered as a prognostic marker [Hausen et al 1981, Piccinini et al 1991]. In patients with multiple myeloma neopterin concentrations were found to be more predictive of prognosis than interleukin-6 (IL-6) [Reibnegger et al 1991].

Though the sensitivity in patients of breast cancer was low, an increased urinary neopterin excretion was found to be a sign of poor prognosis attributed to either disease progression or events leading to death [Reibnegger et al 1987, Murr et al 2002 and Melichar et al 2006a]

Yildirim et al. suggest that neopterin seems to be an indicator of metastatic cancer rather than a marker for local cancer [Yildirim et al 2008]. A review of literature would reveal that increased urinary and serum neopterin concentrations have been reported in patients with gynecological cancer, including epithelial ovarian carcinoma, cervical carcinoma, endometrial carcinoma, uterine sarcomas and vulvar carcinoma [Melichar et al 2006a], colorectal cancer, liver tumors and breast cancer. In all tumor entities a highly significant association was found between neopterin concentrations and the risk for relapse, metastases or death. High neopterin levels have been found to be significantly associated with a poor prognosis of the underlying malignant disease.

Table 2: Depictions of evidence in literature on tumor types and the use of neopterin in prognosis (Table modified from Murr et al, 2002. based on new evidence)

Tumour type	Frequency of increased neopterin in %	Prognostic significance
Hodgkin's disease	70-100	yes
CML	95	no
Ovarian	82	yes
Uterine sarcoma	78	-
Multiple Myeloma	30-70	yes
Pancreatic carcinoma	69	yes
Lung cancer	58	yes
Squamous cell cancer – oral cavity	43	yes
Colon cancer	48	yes
Cervical carcinoma	55	yes
Stomach carcinoma	42	yes
Prostatic carcinoma	25	yes
Malignant melanoma	Below 25	-
Breast carcinoma	18-25	yes
Hepatocellular carcinoma	-	yes

Anticancer therapy, immune activation and neopterin

The effect of anticancer therapy on immune system and potential of immunomodulation in anticancer therapy has been a topic of interest for the oncology community. Several studies have described effects of cytotoxic drugs on macrophages, dendritic cells (DCs) and natural killer (NK) cells. In their experiments with rodents, Ghiringhelli et al. have shown that oral administration of metronomic (low dose) cyclophosphamide in advanced cancer induces a profound and selective reduction of circulating regulatory T cells, associated with a suppression of their inhibitory functions on conventional T cells and NK cells leading to a restoration of peripheral T cell proliferation and innate killing activities [Ghiringhelli et al 2007]. In another study cyclophosphamide increased the frequency of tumor-infiltrating CD4 and CD8 cells containing interferon gamma, NK, dendritic cells, with the greatest increases seen among tumor-infiltrating lymphoid cells (TIL) in mice tumors [Pu et al 2010]. Another chemotherapeutic agent 5-FU in conjunction with IFN- α was found to enhance the NK mediated cytotoxicity [Khallouf et al 2012].

There is evidence that chemotherapy has an effect on macrophages too. Again cyclophosphamide metabolites were found to increase the tissue associated macrophages (TAM-M1) that enhance the production of proinflammatory IL-6, IL-12 and oxygen radicals, and thus can significantly increase the specific immune response as well as nonspecific innate reaction [Brynarski et al 2009]. Paclitaxel can not only stimulate TAMs cytotoxicity directly [Park et al 2013] but also induce the activation of dendritic cells (DCs), NK and tumor-specific CTL via the secretion of IL-12, TNF- α and inducible nitric oxide synthase (iNOS) by TAMs, [Javeed et al 2009] resulting in tumor regression [Bracci et al 2014].

In addition, there have been studies depicting a direct immunomodulatory effect of chemotherapy. In an unbiased functional screen of 54 chemotherapeutic agents, Tanaka et al. unveiled the diversity of the tested drugs on the maturation, survival and growth of DCs [Tanaka et al 2009]. The drugs delivering DC maturation signals at concentrations causing only marginal DC death included topoisomerase inhibitors (for example, etoposide, mitoxantrone, doxorubicin), antimicrotubule agents (for example, vinblastine, paclitaxel, docetaxel) and the two alkylating agents mechlorethamine and diaziquone [Tanaka et al 2009]. In a similar study in human

macrophages the authors were able to demonstrate that in low noncytotoxic concentrations chemotherapeutic agents do not induce apoptosis of DCs, but directly enhance DC maturation and function [Kaneno et al 2009]. In another report, paclitaxel, doxorubicin and methotrexate were shown to promote the ability of murine BM-DCs to present antigens to T-cells in vitro by upregulating antigen-processing machinery gene components, costimulatory molecules and IL-12 [Shurin et al 2009].

In an interesting study it was shown that 5-FU and doxorubicin could induce in vitro cancer expression of heat shock proteins (HSPs) and thereby promote the engulfment of cell debris by human DCs and the subsequent cross- presentation of tumor antigens to T-cells [Bracci et al 2014, Buttiglieri et al 2003]. Thus, it could be argued that the changes in the neopterin levels could reflect the complex immunological play being staged in the system of a patient suffering from malignancy whilst they are on treatment.

Similar to cytotoxic chemotherapy, radiation is known to cause direct tumor damage by the DNA breakdown and by affecting various intracellular process. The net effect is cancer cell death and tumor control. In addition to the direct effect on cancer cells, radiation has also been shown to affect the neopterin levels [Holečková et al 2013] and thus reflect changes in immune system. A study was able to demonstrate that α irradiation can stimulate adaptive immunity, can elicit an efficient antitumor protection, and therefore could be an immunogenic cell death inducer [Gorin et al 2014]. In another study dealing with the impact of high-dose ablative radiotherapy (RT) on tumor microenvironment components, the high dose ablative RT given to the tumor was found to induce bystander/abscopal factors and endothelial cell death coupled with immune activation [Prassanna et al 2014]. Various researchers have tried to use the effects of RT on the immune system for immunotherapy. Witek et al. have argued that optimal sequencing of RT and immunotherapy amplifies antigen-specific local and systemic immune responses, revealing novel acute and long-term therapeutic antitumor protection [Witek et al 2014]. In an observation it was found that whereas high doses of radiation (>10 Gy) can lead to lymphopenia, lower radiation doses (2-4 Gy) represent a valid treatment option in some hematological cancers and it triggered clinically relevant immunological changes. In the same study it was shown that RT might potentially enhance T cell function

induced by synergistic radiation treatment with potential physiological significance in a wide range of T cell responses [Spary et al 2014]. Again, monitoring the neopterin levels may reflect these radiotherapy induced immune changes.

Though we have significant data on neopterin and conventional anticancer treatment, the information of neopterin and targeted biological therapy is limited. In modern oncology practice biological therapy is increasingly playing a crucial role in management of various tumor types leading to an improved treatment outcome. However, not all patients benefit equally from similar biological therapy and the precise mechanisms by which the targeted agent such as cetuximab act appear to be more complex than previously thought.

A good example can be the finding that the EGFR targeted drug did not benefit all patients and it was later revealed that it was due to the KRAS status of the patient. The analysis of pooled data from the CRYSTAL and OPUS studies confirms the consistency of the benefit obtained across all efficacy end-points from adding cetuximab to first-line chemotherapy in patients with KRAS wild-type metastatic colorectal cancer [Bokemeyer et al 2012]. However, activity had also been demonstrated for cetuximab in combination with chemotherapy in the first line of treatment of metastatic colorectal carcinoma in patients with tumors not harboring RAS mutation [Bokemeyer et al 2009, Van Cutsem et al 2009]. Thus, underlining alternative mechanism of action of this agent. With such information the precise mechanism of anti-tumor activity of cetuximab is still being explored. Similar to cetuximab, most of targeted drugs may inhibit tumor growth through more than one mechanism acting on multiple molecular targets. Among proposed mechanisms of biological agents, the activation of the host immune response has also been implicated [Messersmith et al 2007, Zhang et al 2007].

In a study of patients of lung cancer it was found that *in vivo* antitumor activity of cetuximab could be associated with a complement-mediated immune response. In the same study Hsu et al. emphasized the need to elucidate the molecular mechanisms involved in cetuximab activity in order to improve its clinical efficacy and to better select patients who would benefit from cetuximab treatment [Hsu et al 2010]. In a recent article it was proposed that cetuximab can inhibit tumor growth by blocking oncogenic signals and initiating antibody –dependent cellular cytotoxicity

(ADCC), which not only suppresses tumor growth but also triggers innate immunity to improve CTL cross-priming by DC. This enhanced CTL response, in turn, can then kill more tumor cells to create a positive loop that initiates danger/innate signaling that further generates both innate and adaptive immunity against the tumor, and ultimately leads to tumor regression [Xuanming et al 2013].

Collectively, these studies provide insight into a novel antitumor mechanism of anti-EGFR Ab therapy that promotes cooperation between innate and adaptive immunity and warrants reconsideration of the adaptive immune system in current therapy regimens and antitumor therapy design [Xuanming et al 2013]. Moreover, as presented in a review, anticancer drugs such as fluorouracil and gemcitabine administered prior to mAb administration could induce antigen reediting (induction of neoantigens and/or upregulation of pre-existing antigens) in cancer cells and activate powerful danger signals (i.e., HSP-90 and calreticuline). Once opsonized and/or phagocytosed by DCs and macrophages these cells could become a great source of neoantigens available for an efficient antigen-specific T-cell response with long-term memory [Correale et al 2011].

The figure below can explain the possible role of targeted agent (cetuximab) in immune modulation.

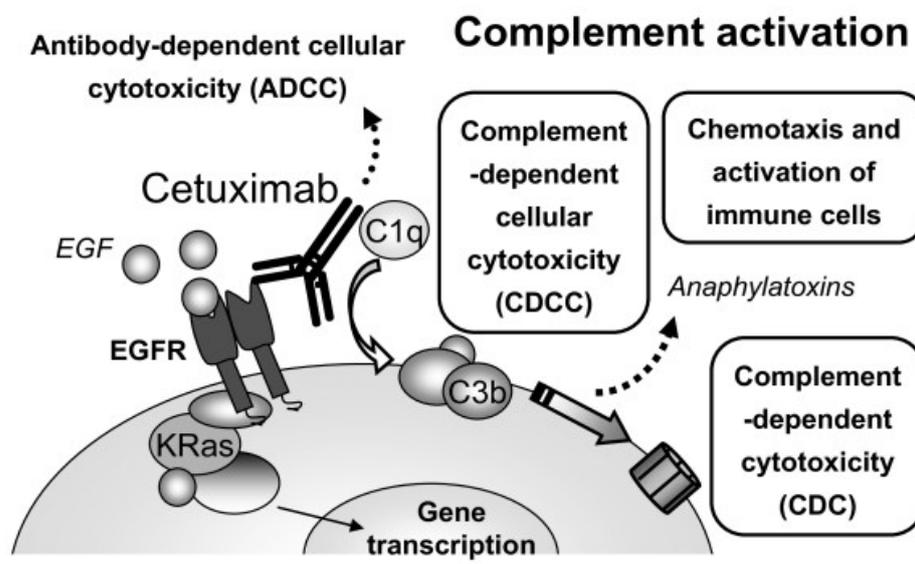


FIGURE 6: The proposed immune modulatory mechanism of targeted agent (cetuximab) [Correale et al 2011]

The immunological activity associated with administration of biological agents will be a matter of great interest with the advent of newer immunomodulatory drugs. Based on the above evidence it could be argued that the biomarkers of immune activity can play a vital role in monitoring the response to therapy. It can also provide more information in the pursuit to explain the anti-cancer mechanism of targeted therapy. Neopterin by virtue of reflecting systemic immune activation could be an important tool to investigate not only mechanism of action of biological agents but could also help in identifying the subtypes with varying degree of response.

The levels of neopterin are also linked to several other parameters that may directly influence carcinogenesis, response to treatment and even complication in cancer patients. The association of several clinical and laboratory parameters with neopterin has been proven by both laboratory and clinical studies. A review of some major factors can provide an insight into the potential use of measuring neopterin levels in cancer patients.

Neopterin and weight-loss and cachexia

Clinically the presence of cachexia has been shown to be a significant risk factor and an indicator of negative prognosis in cancer patients [Ramos et al 2004]. Unfortunately, there is a dearth of strong evidence based management strategy for cancer related cachexia. Cachexia is essentially a catabolic state that is mediated by multiple factors. Factors like TNF-alpha {cachectin} and IFN- gamma may work individually or in conjunction leading to the breakdown of the tissues [Moldawer et al 1997]. Kurzrock has highlighted the roles of different interleukins in addition to TNF, IFN-gamma, and leukemia inhibitory factor acting as cachectins in animal models [Kurzrock et al 2001].

In their study with patients with HIV infection Zangerle et al found weight loss of more than 10% of body weight is associated with immune activation [Zangerle et al 1993]. They observed a correlation between body mass index, urinary neopterin and CD4+ T-cell count, development of AIDS-defining infections, weight loss, and a decline in CD4+ T-cell [Zangerle et al 1993]. Extrapolating from their result one could hypothesize that the neopterin levels can reflect the pro inflammatory states in cancer patients and can potentially be used to monitor the progression or development

of cancer cachexia and other complications. Serial monitoring of neopterin levels in cancer patients could potentially forewarn clinician of processes leading to this complication. In future a better understanding of the biochemistry of cachexia could provide more insight into development of this complication and we may be able to adopt measures to prevent and treat this symptom and improve the quality of life of the patients.

Neopterin and Anemia

Anemia is a common finding in cancer patients and a correction of anemia plays a vital role for optimal treatment outcome. Neopterin levels were found to be elevated in cancer related anemia [Fuith et al 1989, Weiss et al 2004] and an inverse correlation of the urinary neopterin levels with hemoglobin levels has been found in patients [Fuith et al 1989, Denz et al 1992].

Further studies corroborated the early findings of a negative correlation between urinary neopterin and hemoglobin, hematocrit, serum iron, iron-binding capacity, and transferrin saturation indexes whereas a positive correlation was observed between urinary neopterin and serum ferritin and erythropoietin levels [Ji et al 2012]. These results implicate the systemic immune activation, reflected in increased neopterin production, in the pathogenesis of anemia in patients with cancer [Melichar et al 2006a].

Neopterin, depression and fatigue

Patients with malignant disease can present with profound fatigue, severe mood changes and depression. Enhanced neopterin production leads to deficient BH₄, which is critically involved in the biosynthesis of biogenic amines including serotonin and several adrenergic/dopaminergic neurotransmitters [Neurauter et al 2008]. Thus neopterin may reflect the immune mechanism of various cancer related neuropsychiatric symptom. For example increase in urinary neopterin levels were found to precede increase in fatigue intensity with a temporal delay of 60—72 hours [Haberkorn et al 2013].

Potential use of neopterin in detecting complications

Neopterin can also reflect the systemic immune and inflammatory responses in various other human disorders. A review of literature reveals that neopterin has been employed to study a range of pathologies.

Studies on patients with clinical evidence of infection and related reviews have tried to highlight the utility of neopterin in these cases [Cesur et al 2005]. For example neopterin has been investigated for use in monitoring sepsis and tuberculosis [Turgut et al 2006, Berdowska et al 2001]. Neopterin levels have also been shown to have meaningful trends in HIV infection [Melichar et al 2006a], parasitic infection [Berdowska et al 2001] and other exotic infections [Handan et al 2005]. In addition to reflecting the disease states neopterin has also been employed to monitor disease process and response to treatment. For example, some findings suggest that the pretreatment level of neopterin might be used in routine clinical practice as rapid and cost-effective marker to predict the response to antiviral therapy in HCV patients [Oxenkurg et al 2012]. Neopterin measurements were also useful to monitor therapy in patients with HIV infection [Murr et al 2002].

In addition, neopterin has also been used to monitor therapy in autoimmune disorders. It has been suggested that determination of tryptophan degradation and neopterin levels in chronic inflammatory disease may provide a better understanding of progression of these diseases [Ozkan et al 2012].

Neopterin has also garnered interest from workers studying ageing as it is also supposed to represent a characteristic of ageing. Immune deviations that are most widely expressed in the elderly include increased neopterin production and tryptophan breakdown. Increases in neopterin were found to correlate with a substantial decline in key vitamins, including folate and vitamin-B6, -B12, -C, -D and -E. [Capuron et al 2014]. Interestingly, a correlation between baseline parameters of intestinal permeability and urinary neopterin has also been observed [Dvorak et al 2010].

A remarkable use of neopterin has been made in investigations of acute medical conditions too. Schumacher et al. study produced data to support the hypothesis of an activation of monocytes and macrophages in patients with an acute or chronic coronary syndrome. They suggested that neopterin is significantly

increased in patients with chronic coronary artery disease and more pronounced in patients with acute myocardial infarction shortly after the onset of symptoms [Schumacher et al 1997]. A prospective study suggested that determinations of neopterin and IL-18 concentrations might represent early markers for post-traumatic complications such as multiple-organ dysfunction syndrome and sepsis [Mommensen et al 2009].

There are several other noteworthy studies that emphasize the relevance of neopterin in clinical investigations. For example screening of neopterin concentrations in blood donations allows detection of acute infections in a non-specific way and improves safety of blood transfusions [Murr et al 2002]. In a study on patients with kidney transplant by Carey et al, higher neopterin levels were correlated with acute rejection in the first year post-transplant, but this was only significant in recipients who received kidneys from donors after cardiac death, and who suffered acute cellular or vascular rejection [Carey et al 2013].

Neopterin in therapy related complications

Not only was neopterin found to depict the immune activation due to therapy but due to its peculiarity it also rose in other inflammatory condition including the complications that frequently occur in cancer patients. An association of complications and higher levels of neopterin have been documented and urinary neopterin concentrations were found to be relatively stable in cancer patients in the absence of complications [Melichar et al 2007a]. Significantly higher neopterin levels were noted in patients with two or more comorbid conditions. Data by Melicharova et al. has demonstrated an association between systemic immune activation, reflected in increased urinary neopterin concentrations, and age or presence of comorbid diseases in patients with breast carcinoma. A cumulative effect was observed with the presence of two or more comorbid conditions resulting in significantly increased urinary neopterin [Melicharova et al 2010]

In a noteworthy study on patients of head and neck tumors neopterin level was significantly increased following the changes of the intensity of nausea, vomiting, mucositis, skin toxicity, xerostomia, laryngeal toxicity, pharyngeal toxicity, upper gastrointestinal tract toxicity and performance status. The changes of urinary

neopterin that resulted from changes of toxicity were delayed by lag times ranging from 5 to 24 days. The strongest effect on neopterin concentrations was observed for nausea, mucositis and performance status [Holečková et al 2013]. Administration of anticancer therapy induces an inflammatory response. Changes in physiology associated with systemic inflammatory response, including metabolism of trace elements and vitamins, may play an important role in the toxicity of combined-modality treatment [Holečková 2013]. In addition, earlier in section on neopterin in human pathology, we have seen the association of neopterin in non-malignant condition, too. Thus, the ability of neopterin to reflect the immune outcome of variety of clinically relevant conditions can potentially be utilized in monitoring complication in cancer patients.

Thus one could argue that neopterin being a marker of immune activation, could possibly be used in clinical settings to monitor immune activity in cancer patients. Immune activity may be responsible for overall outcome, response to treatment and may even reflect the complication. With the background of above evidence we sought to test the potential use of neopterin in prognosis, monitoring response to various anticancer therapies and monitoring complication in cancer patient.

4. Objective

Based on the available evidence we hypothesized that neopterin has the potential to be of use in routine oncology practice specifically in the assessment of prognosis, monitoring response to therapy and complications in cancer patients.

To test the hypothesis a project was designed to investigate specific aspects mentioned above. The project addressed the associations of neopterin and the following clinically relevant issues.

1. The changes in neopterin levels during anticancer therapy including chemotherapy, biological therapy and radiotherapy.
2. Utility of neopterin in predicting prognosis
3. Correlation of neopterin with various laboratory parameters
4. The correlation of neopterin and complications in patients undergoing anticancer treatment

Venue

The study was mainly performed at the University Hospital in Hradec Kralove.

The patients being treated at the department on oncology and radiotherapy were recruited for the study.



Department of Oncology and Radiotherapy

University Hospital, Hradec Kralove

5. Study design

Our project was divided in two parts

Part I

To analyze urine samples in patients to investigate if neopterin levels could be used in prognosis and monitoring response to systemic and biological therapy.

Part II

To analyze the urinary neopterin in patients during chemo-radiotherapy and to investigate association of neopterin to complications

For the purpose of our study, different cohorts of patients were recruited:

1. Cohort A- Patients of Metastatic colorectal carcinoma undergoing systemic treatment, n=45
2. Cohort B -Patients with Gynecological malignancy undergoing pelvic radiotherapy with concomitant chemotherapy, n=10

The total number of patients in whom urinary neopterin was studied was 55.

The individual patient groups, characteristics, methodology and results relevant to the study parts are described separately.

Sets of patients and statistical analysis

The investigations were carried out at the University Hospital in Hradec Kralove. The patients were enrolled at the Department of Oncology and Radiotherapy and the sample were stored in the specially assigned refrigerators in the Department of Oncology and also at the Department of Pathology.

The determination of urinary neopterin was performed in the laboratory of the Third Department of Medicine – Gerontology and Metabolic Care.

In the following sections we present:

- Patient characteristics in all patient groups
- Methodology
- Statistics
- Results

All aspects of study in individual patient cohorts have been presented separately.

6. Study

6.1 Part I

6.1.1 Patient group- Cohort A

The patient group was selected from the patients being treated at the University Hospital in Hradec Kralove

Diagnosis of patients in cohort A

Metastatic colorectal carcinoma

Patient characteristics

- Number of Patients = 45
- Male 28
- Female 17
- Aged (mean±standard deviation) 60±11 (range 32–78)

Anticancer therapy in patients of cohort A

43 Patient – Treated with

Cetuximab (loading dose 400 mg/m², subsequently 250 mg/m² weekly) followed by **irinotecan** (180 mg/m²), leucovorin (200 mg/m²), and **5-fluorouracil** (400 mg/m² bolus and 1200 mg/m² for 46 hours) . The regimen was administered two weekly.

1 Patient – treated with

Cetuximab (loading dose 400 mg/m², subsequently 250 mg/m² weekly) followed by **irinotecan** (180 mg/m²), leucovorin (200 mg/m²), and **5-fluorouracil** (400 mg/m² bolus and 1200 mg/m² for 46 hours) . The regimen was administered two weekly with modification. (Irinotecan was omitted due to hypebilirubinemia)

1 Patient – Treated with cetuximab monotherapy

Summary of patient group A

Forty-five consecutive patients with metastatic colorectal carcinoma, 28 males and 17 females, aged (mean±standard deviation) 60±11 (range 32–78) years were included in the study. Forty-three patients were treated with the combination of cetuximab (loading dose 400 mg/m², subsequently 250 mg/m² weekly) followed by irinotecan (180 mg/m²), leucovorin (200 mg/m²), and 5-fluorouracil (400 mg/m² bolus and 1200 mg/m² for 46 hours) every two weeks (including one patient who received a modification of this regimen). One patient with hyperbilirubinemia had been treated with the above regimen omitting irinotecan, and one patient was treated with cetuximab monotherapy. All patients had been previously treated with oxaliplatin, and all but one patient had been pre-treated with an irinotecan-containing regimen.

6.1.2 Methodology

All patients were being treated at the Department of Oncology and Radiotherapy at the University Hospital in Hradec Kralove.

The investigations were part of a project approved by the institutional ethics committee and the patients signed informed consent. After consenting, a collection of urinary samples was initiated in all patients.

Early morning urine specimens were collected for each patient before and during the course of anticancer therapy. The first sample was always before the start of treatment. A sterile collection dish was given to the patient and sample was collected from the dish to a sterile urine vial. The sample were labeled and numbered. These vials were immediately transferred into a refrigerator. A temperature of -20^o C was maintained throughout. The entire batches of samples of each patient were serially collected in a separate box until analysis.

The determination of urinary neopterin was performed in the laboratory of the Third Department of Medicine – Gerontology and Metabolic Care.

Sample preparation

The samples were gradually thawed from their frozen state. After a brief centrifugation for 45 second at 12000 x g, 100 ml of urine samples were diluted with 1.0 ml of mobile phase containing disodium-EDTA (2 g per liter), the samples were filtered using Microtiter, AcroPrep 96 Filter Plate 0.2 μm / 350 μL , Pall Life Science (Ann Arbor, MI, USA) and Vacuum manifold Pall Life Science and then injected into a column.

Neopterin was determined using high performance liquid chromatography system Prominence LC20 (Shimadzu, Kyoto, Japan) composed from Rack changer/C - special autosampler for micro titration plates, Degasser DGU-20A5, 2 Liquid chromatograph Pumps LC-20 AB, Auto sampler SIL-20 AC, Column Oven CTO – 20 AC Thermostat, Fluorescence detector RF- 10 AXL, Diode array detector SPD – M20A and communications bus module CBM-20A. Phosphate buffer 15 mmol/L, pH 6.4 with flow rate 0.8 mL/min was used as mobile phase. Separation was performed using hybrid analytical column Gemini Twin 5 μ , C18, 150 \times 3 mm (Phenomenex, Torrance, CA, USA) at 25°C, injection volume was 1 μL . Neopterin was identified by its native fluorescence (353 nm excitation, 438 nm emission wavelength).

Creatinine was monitored simultaneously in the same urine specimen with diode array detector at 235 nm. Time of analysis for urine neopterin and creatinine was 6 minutes and the analytes were quantified by external standard calibration. The neopterin concentrations were expressed as neopterin/creatinine ratio ($\mu\text{mol/mol}$ creatinine).

Hemoglobin was measured by a photometric method using sodium lauryl sulfate, leukocytes and platelets were determined by impedance method using a Sysmex XE-2100 blood analyzer (Sysmex, Kobe, Japan).

Differential leukocyte count was obtained.

Serum carcinoembryonic antigen (CEA) was determined by radioimmunoassay using a commercial kit (Immunotech, Prague, Czech Republic)

Neopterin cut off

Normal values and upper limits of tolerance of urinary neopterin concentrations in healthy persons would change depending on age and sex which is mainly due to variations of urinary creatinine concentrations [neopterin.net]. These variations need to be accounted for when performing study like ours. In our study neopterin levels of 214 ($\mu\text{mol/mol}$ creatinine) was chosen cut off between two groups. This was also found to be upper limit of normal in pervious study by our group [Melichar et al 2008b] and was selected based on medians of respective parameters in the studied group.

Cohort A samples

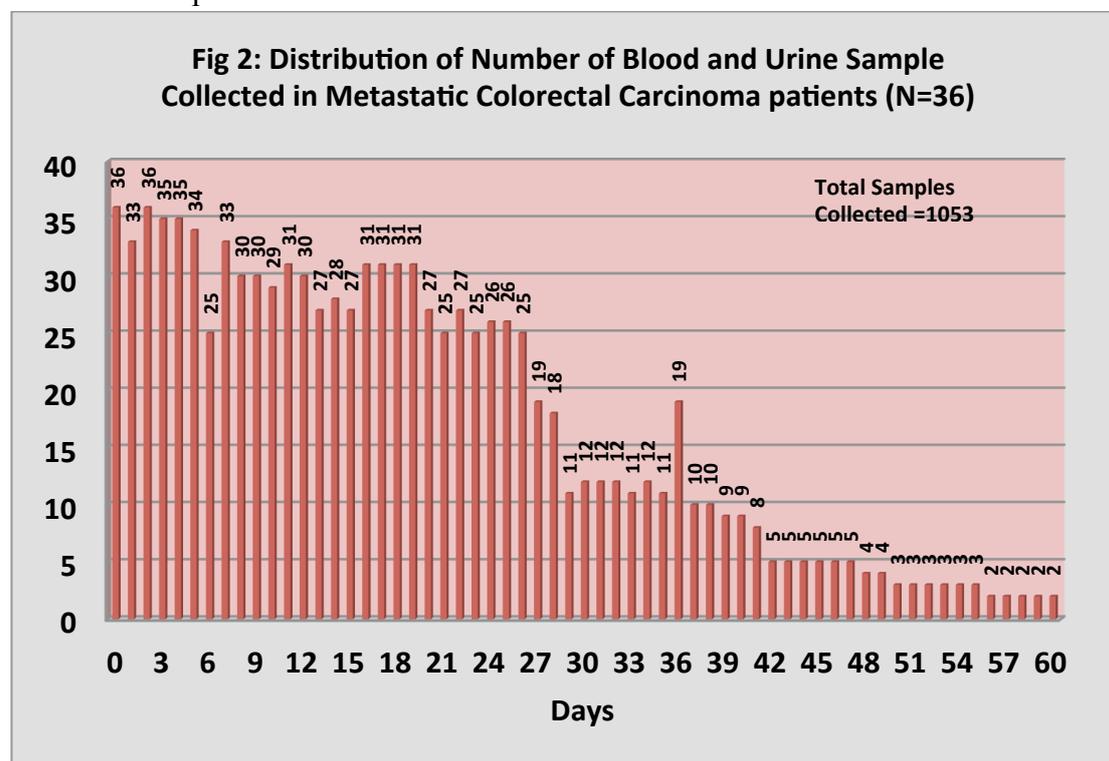


FIGURE 7. Number of serial samples collected in Part 1 of the study with patients in Group A.

Figure 7 shows the sample number collected serially during the treatment in study group A

6.1.3 Statistical tests used

The statistical analysis of the data was performed using NCSS software (Number Cruncher Statistical Systems, Kaysville, Utah, USA).

The following tests were employed

Differences during therapy were evaluated using the Wilcoxon paired test.

Correlations were examined using Spearman's rank correlation coefficient.

Survival was analyzed using the Kaplan-Meier method, and differences were evaluated by log-rank test.

The decision on statistical significance was based on $p = 0.05$ level.

6.1.4 Results

Pretreatment urinary sample was collected in 45 patients

The serial urine collection was completed in 36 patients.

The patients were divided into two groups:

1. Those with initial urinary neopterin concentration of 214 [$\mu\text{mol/mol creatinine}$] or above
2. Those with initial urinary neopterin below 214 [$\mu\text{mol/mol creatinine}$]

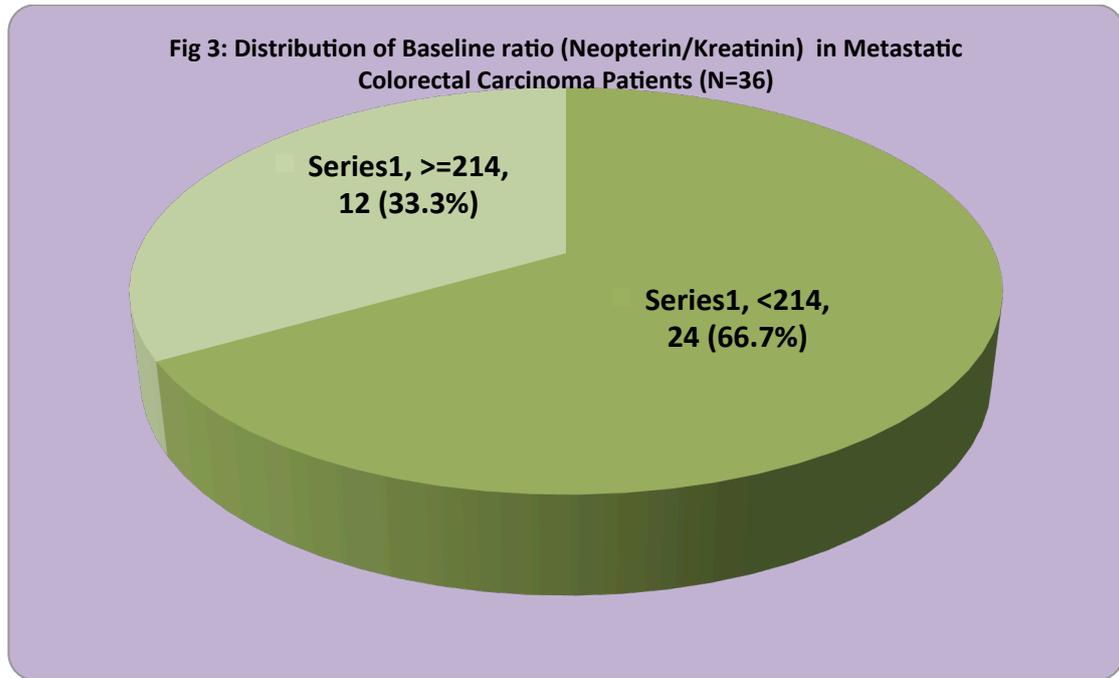


FIGURE 8. Patients of cohort A in whom serial urinary samples were collected were divided in two groups based on High and Low Neopterin/Creatinine ratio (214 as the cut off) are shown in Figure 8

	n	Urinary neopterin ($\mu\text{mol/mol}$ creatinine)				
		Pre-treatment	Peak week 1	Before 2nd cetuximab dose	Peak week 2	Before 3rd cetuximab dose
Whole group	36	251 \pm 232	349 \pm 161 ^c	263 \pm 186	345 \pm 209 ^b	277 \pm 139
Pre-treatment neopterin below 214 $\mu\text{mol/mol}$ creatinine	24	138 \pm 53	299 \pm 115 ^d	194 \pm 80	280 \pm 124 ^c	211 \pm 83 ^a
Pre-treatment neopterin equal or above 214 $\mu\text{mol/mol}$ creatinine	12	477 \pm 287	449 \pm 197	416 \pm 259	480 \pm 286	373 \pm 150

TABLE 3 . The mean \pm standard deviation of neopterin levels in the two groups ^a $p < 0.05$, ^b $p < 0.01$, ^c $p < 0.001$, ^d $p < 0.0001$. The standard deviations of neopterin in high and low neopterin level groups are shown in table 3

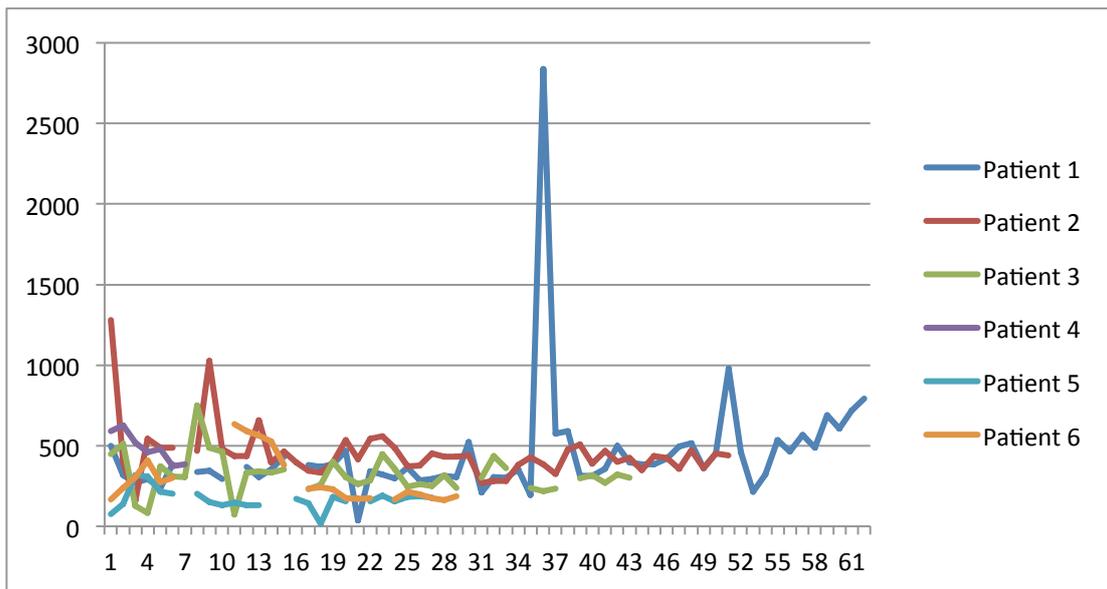


FIGURE 9. The changes in urinary neopterin concentrations during the course of therapy in patients 1-6 of cohort A are shown in Figure 9

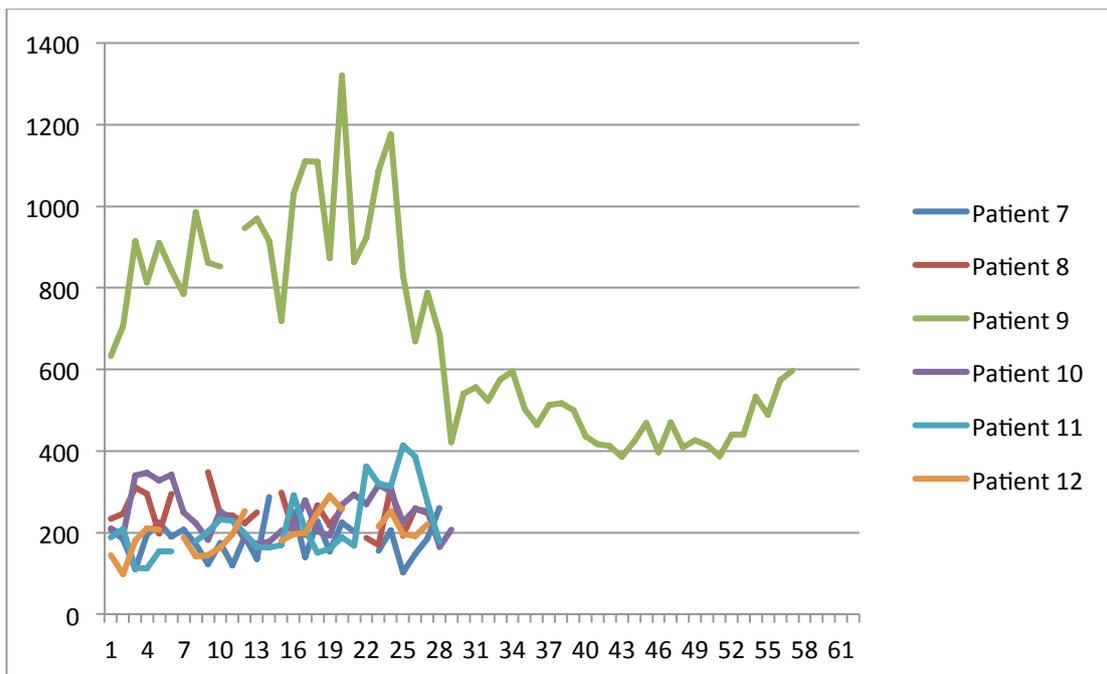


FIGURE 10. The changes in urinary neopterin concentrations during the course of therapy in patients 7-12 of cohort A are shown in Figure 10.

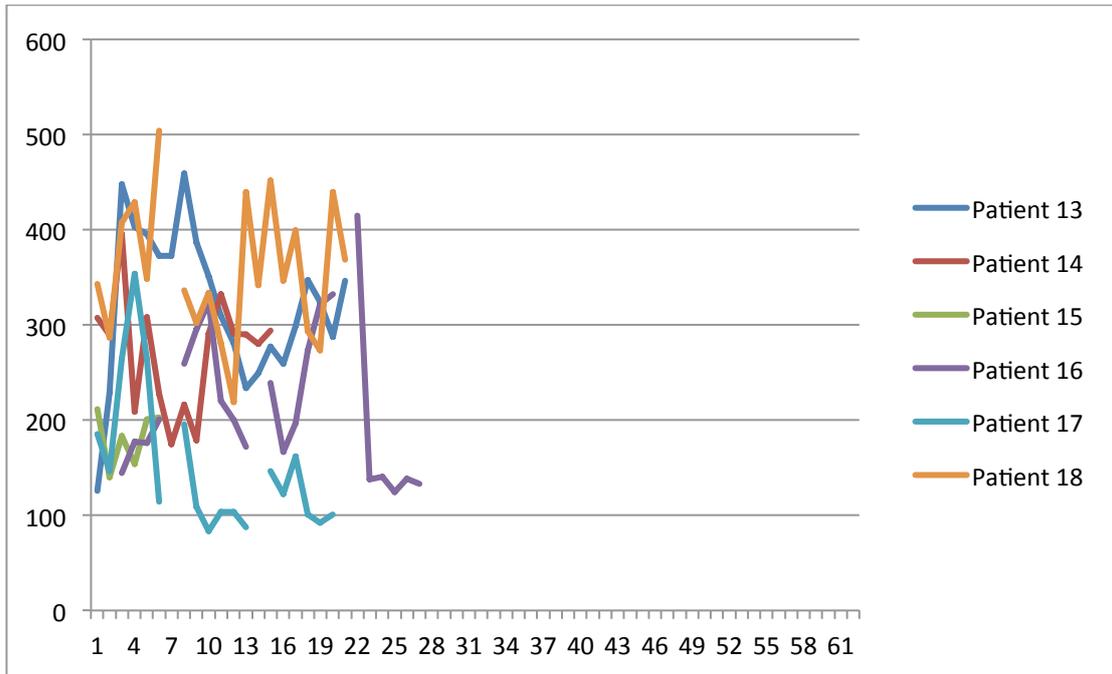


FIGURE 11. The changes in urinary neopterin concentrations during the course of therapy in patients 13-18 of cohort A are shown in Figure 11.

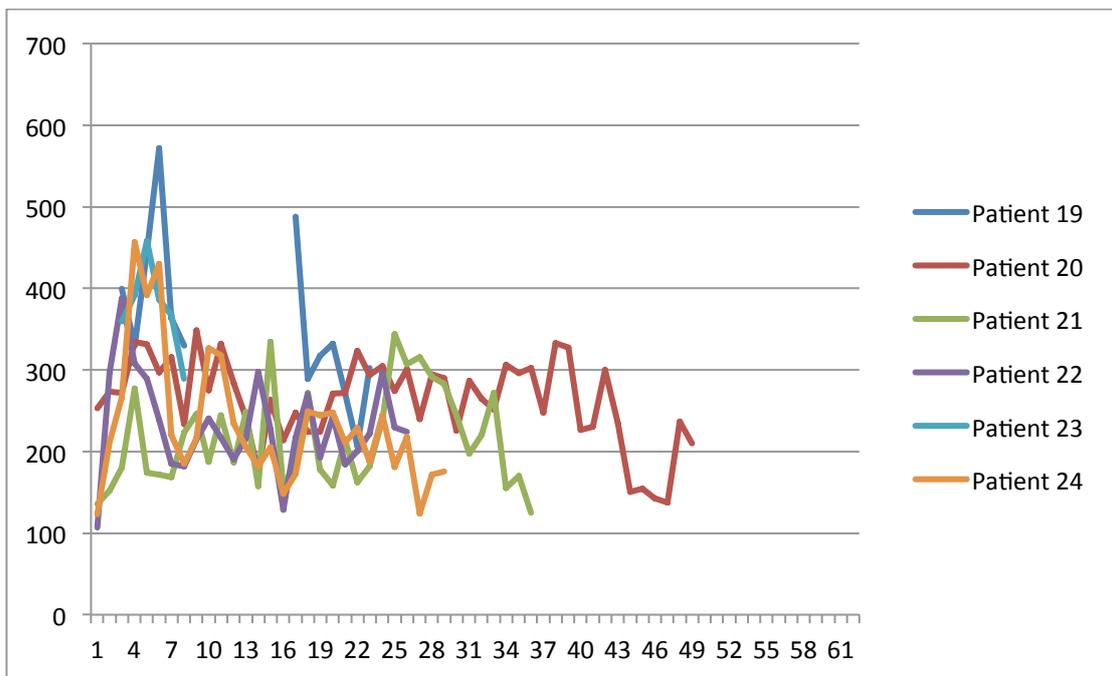


FIGURE 12. The changes in urinary neopterin concentrations during the course of therapy in patients 19-24 of cohort A are shown in Figure 12.

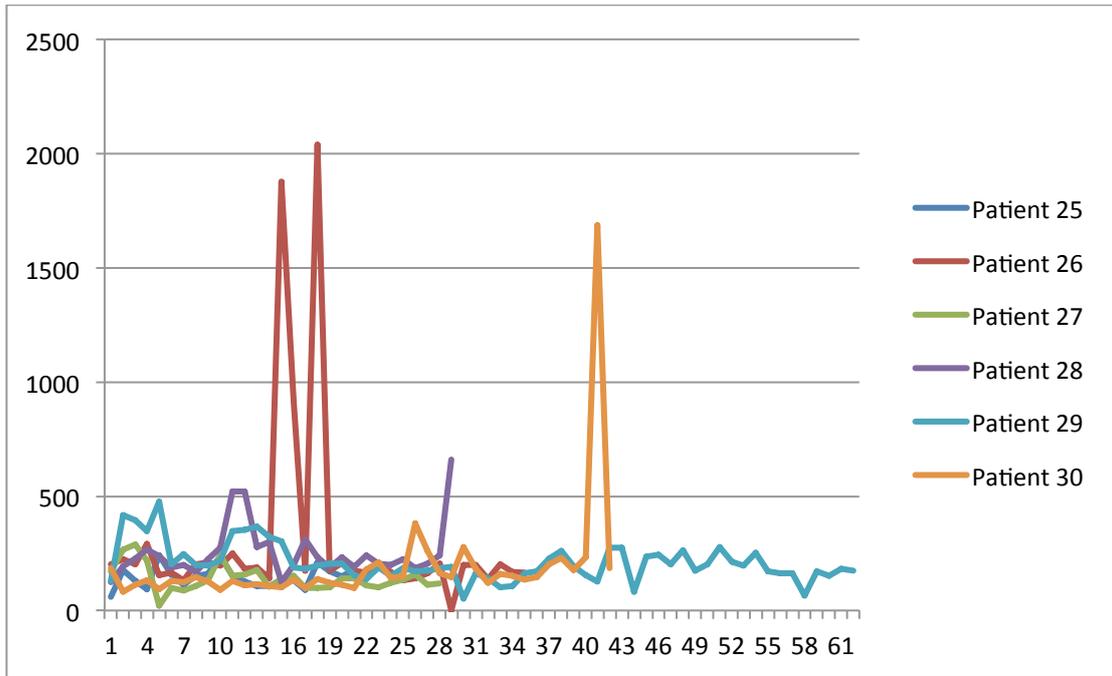


FIGURE 13. The changes in urinary neopterin concentrations during the course of therapy in patients 25-30 of cohort A are shown in Figure 13.

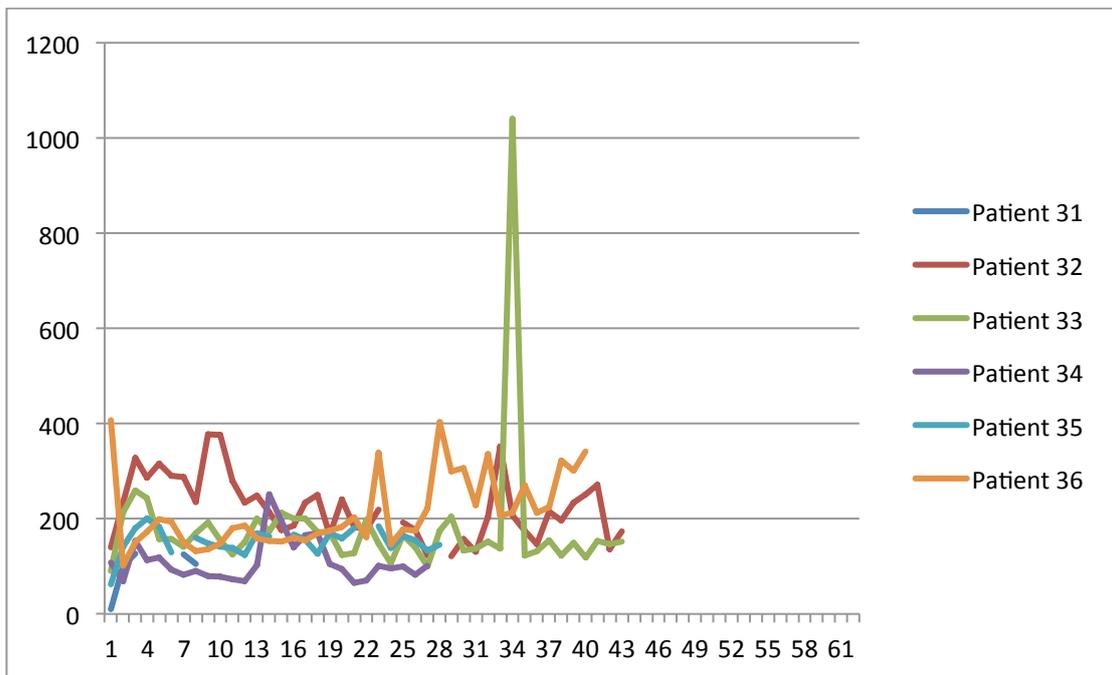


FIGURE 14. The changes in urinary neopterin concentrations during the course of therapy in patients 31-36 of cohort A are shown in Figure 14.

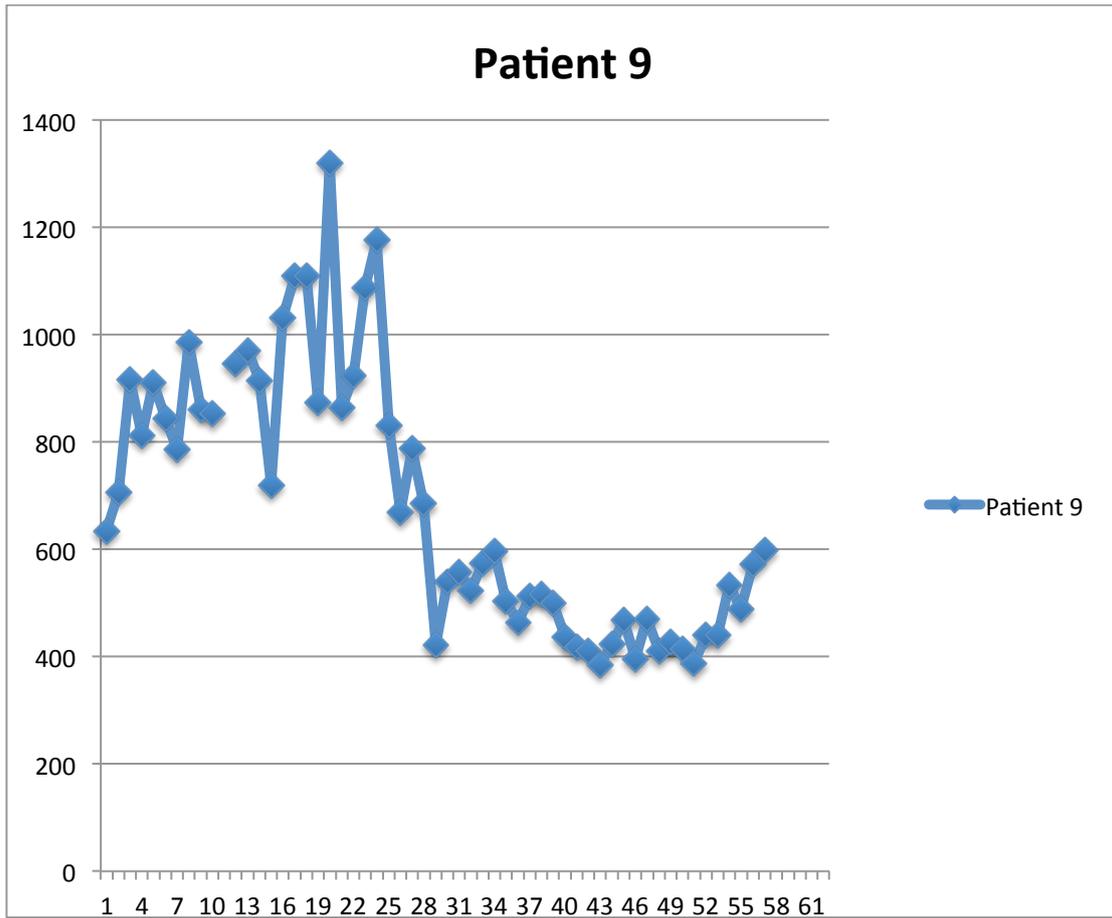


FIGURE 15.

The course of urinary neopterin concentrations during the course of therapy in a 78-year-old patient (patient no 9 of cohort A) treated with single-agent cetuximab is shown above in Figure 15.

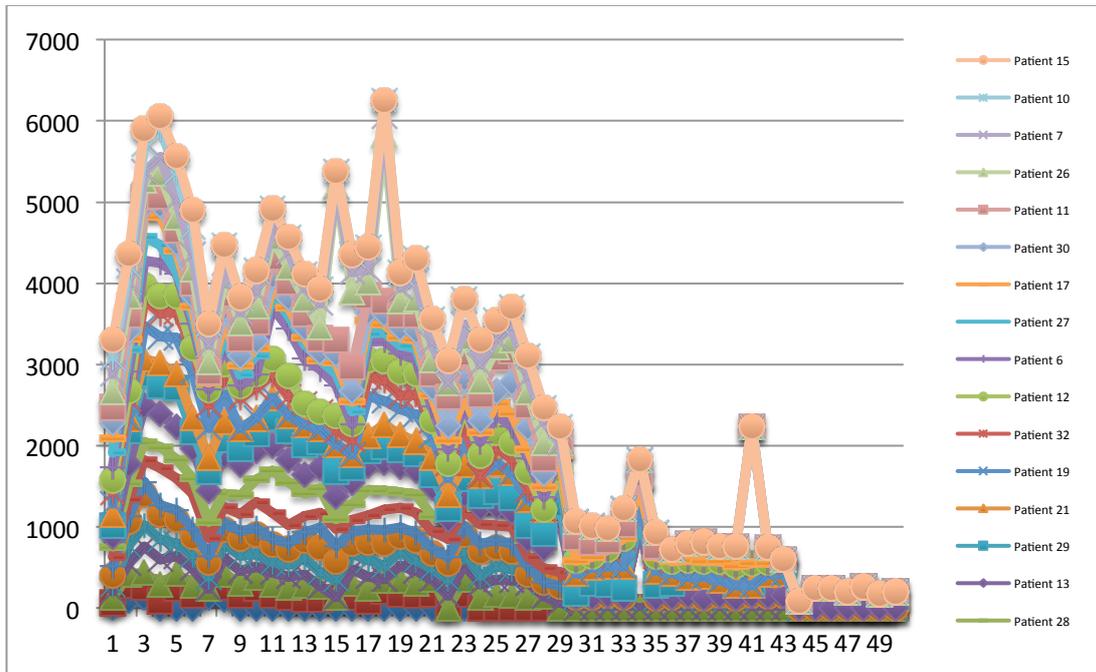


FIGURE 16. Stacked line graph showing trends in patients with Neopterin/ Creatinine < 214 ($\mu\text{mol/mol}$ creatinine) in Group A. The stacked lines showing trend of changes in urinary neopterin levels in patients with pretreatment neopterin < 214 ($\mu\text{mol/mol}$ creatinine) are shown in Figure 16. A general rise in neopterin levels was noted on commencement of anticancer therapy.

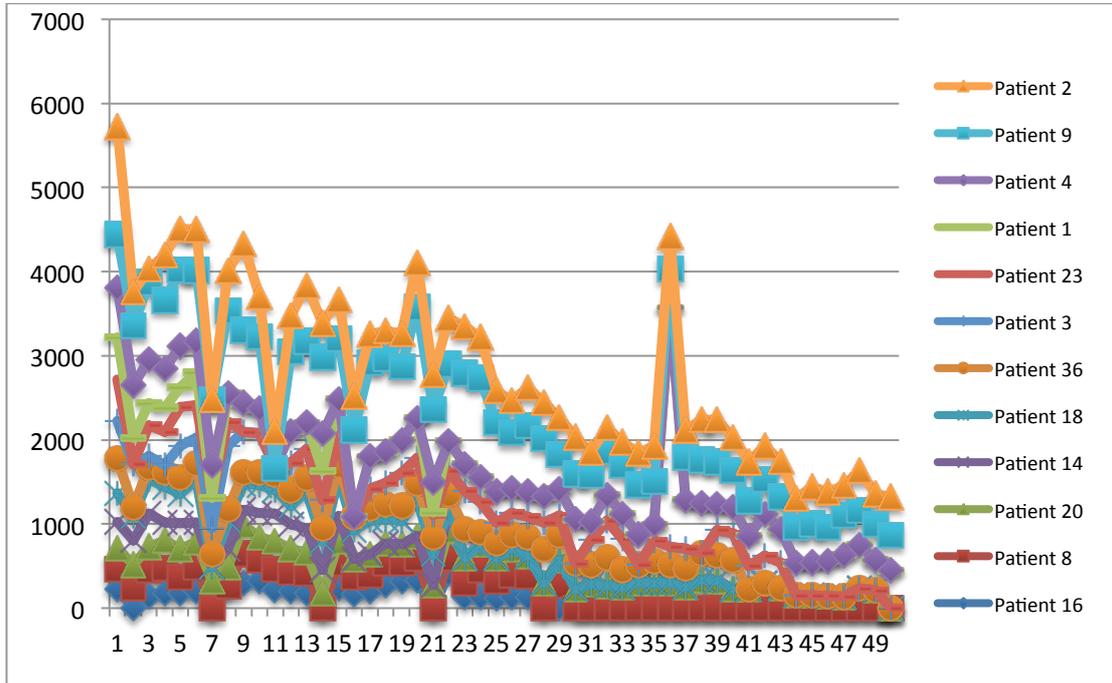


FIGURE 17. Stacked line graph showing trends in patients with Neopterin/ Creatinine ≥ 214 ($\mu\text{mol/mol}$ creatinine) in Group A. The stacked lines showing trend of changes in urinary neopterin levels in patients with pretreatment neopterin ≥ 214 ($\mu\text{mol/mol}$ creatinine) are shown in Figure 17. A general fall in neopterin levels was noted on commencement of anticancer therapy.

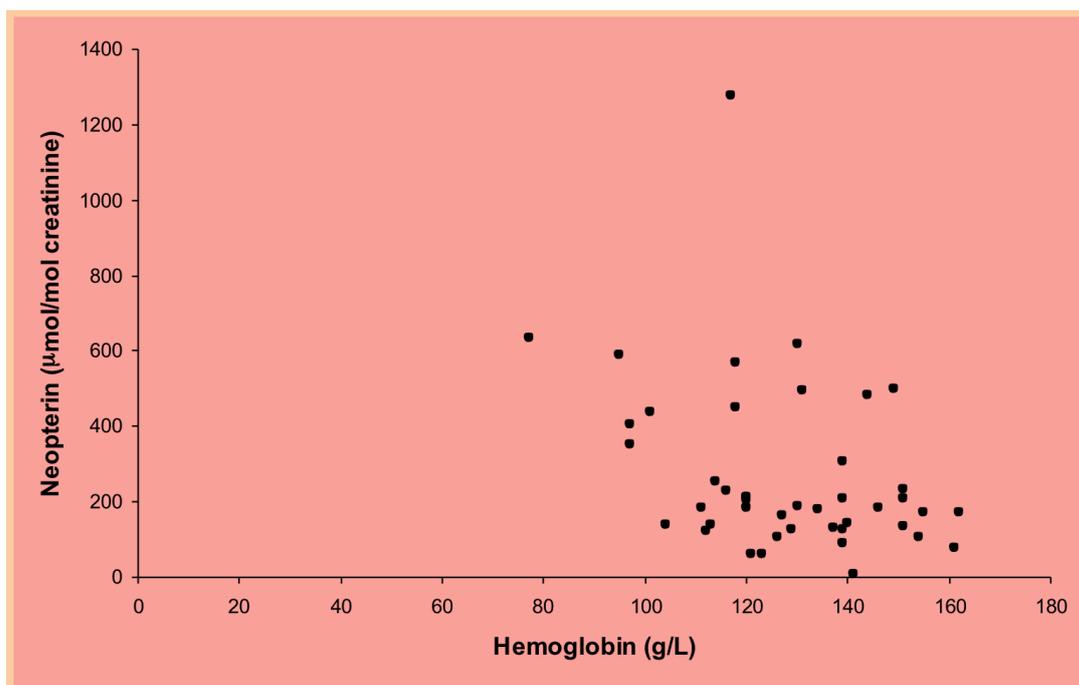


FIGURE 18. Correlation between urinary neopterin and hemoglobin in patients of group A

The negative correlation between hemoglobin and urinary neopterin concentrations ($r_s = -0.34$; $p < 0.05$) is shown above.

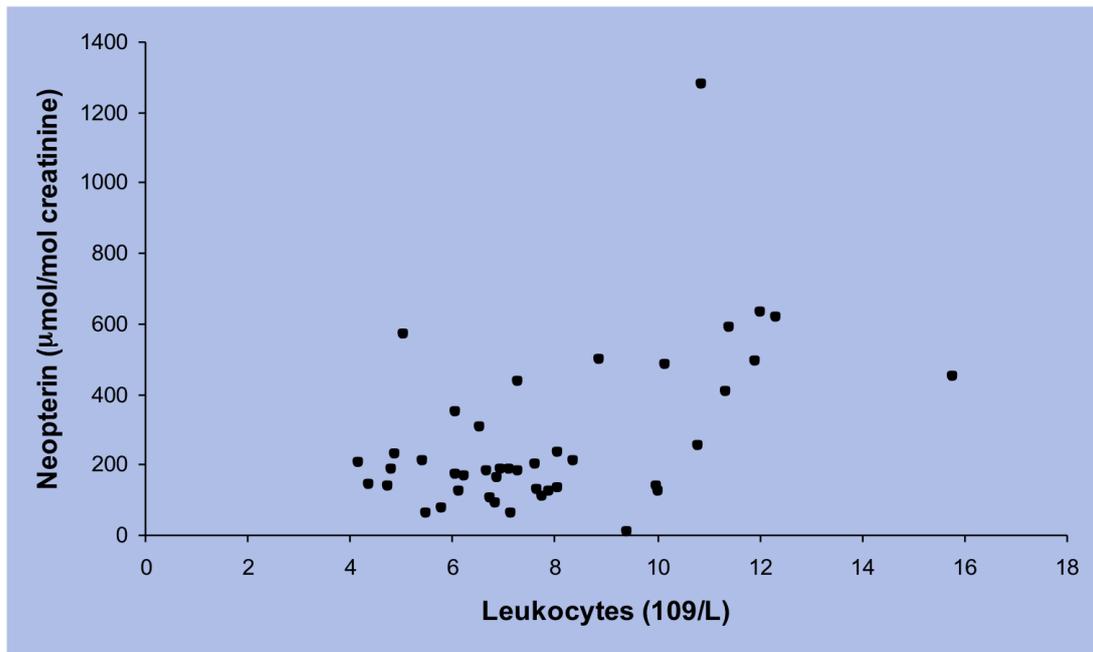


FIGURE 19. Correlation between peripheral blood leukocyte count and urinary neopterin concentrations in patients of group A

The correlation between urinary neopterin and peripheral blood leukocyte count ($r_s = 0.38$; $p < 0.05$) is shown above.

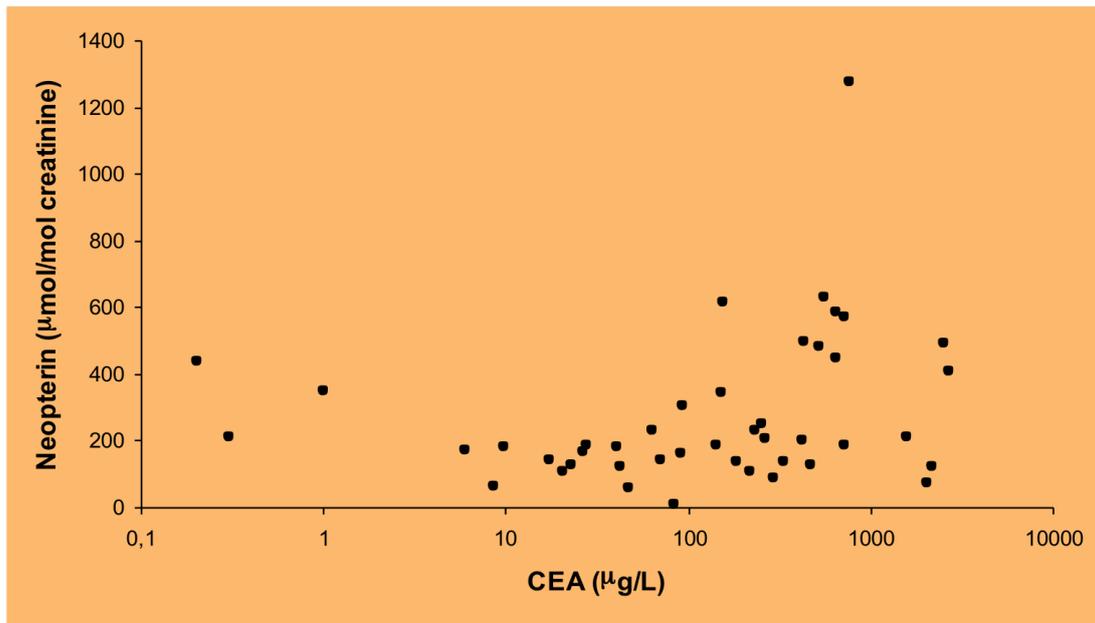


FIGURE 20. Correlation between carcinoembryonic antigen and urinary neopterin concentrations

The correlation between serum carcinoembryonic antigen (CEA) and urinary neopterin concentrations ($r_s = 0.33$; $p < 0.05$) in cohort A is shown.

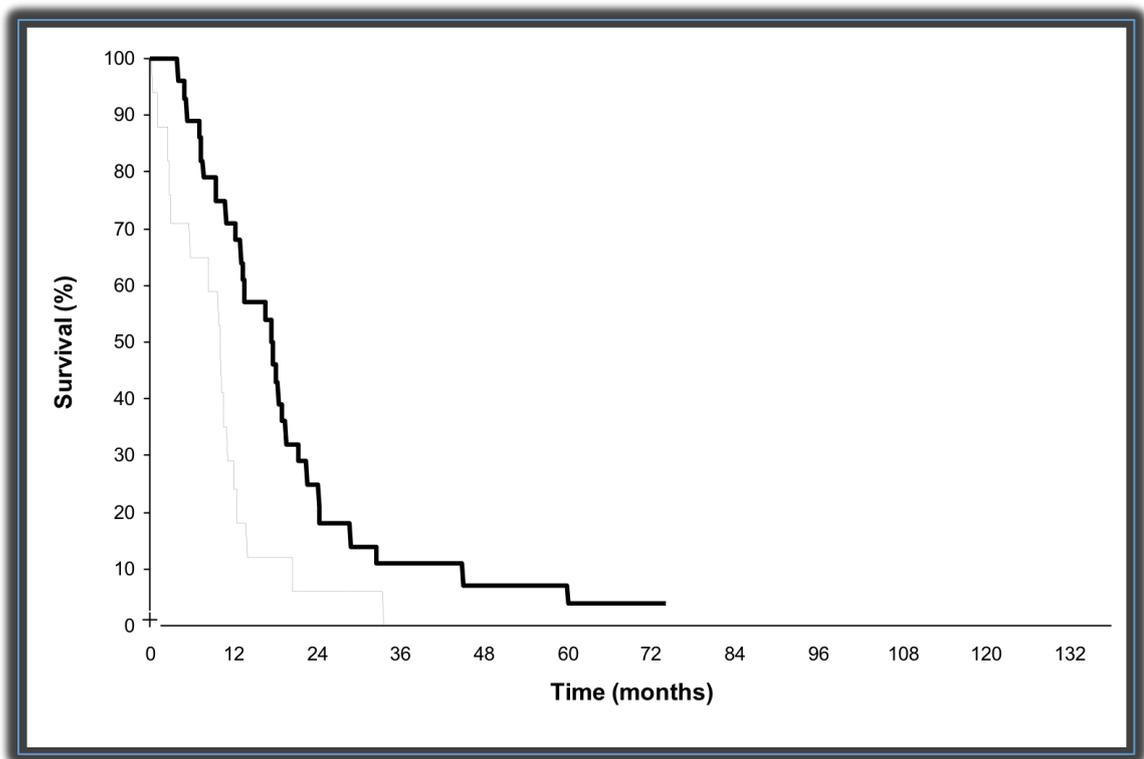


FIGURE 21. Survival curves cohort A

At the time of the analysis, 44 patients died while one patient was alive after 74 months. Survival of patients with urinary neopterin concentration of 214 [$\mu\text{mol/mol creatinine}$] or above was significantly inferior compared to patients with initial urinary neopterin below 214 [$\mu\text{mol/mol creatinine}$] (median 10.1 vs. 17.7 months, $p < 0.05$; Figure 21).

Cohort A = Metastatic colorectal carcinoma

Table 4a

Day	N	Mean	Median	Minimum	Maximum	Std. Deviation
1	36	251	186	10	1279	232.384
2	33	246	214	69	707	146.501
3	36	276	266	110	915	154.888
4	35	293	292	85	813	145.139
5	35	293	243	94	911	153.320
6	34	277	216	93	843	158.300
7	25	239	199	83	786	147.054
8	33	257	202	91	986	182.854
9	30	272	206	79	1025	207.839
10	30	262	240	78	853	150.483
11	29	242	229	72	635	129.842
12	31	260	200	69	946	174.018
13	30	265	211	87	970	186.021
14	27	271	249	106	914	167.414
15	28	323	233	101	1876	332.719
16	27	255	188	122	1031	218.205
17	31	248	199	91	1110	183.827
18	31	313	243	99	2040	364.039
19	31	239	193	92	873	144.442
20	31	271	234	94	1320	220.741
21	27	235	191	37	863	154.604
22	25	259	200	70	922	172.548
23	27	265	204	101	1087	193.473
24	25	261	206	96	1177	212.697
25	26	236	193	100	831	146.858
26	26	238	196	82	669	119.428
27	25	229	206	99	788	141.517
28	19	258	204	119	684	135.629
29	18	250	222	0	660	149.821
30	11	282	245	52	540	158.928
31	12	239	205	131	557	114.164
32	12	260	243	122	523	125.643
33	12	267	262	101	574	126.124
34	11	334	212	108	1040	272.916
35	12	238	185	122	503	119.111
36	11	467	212	125	2839	794.343
38	19	314	248	123	591	146.314
39	10	300	298	149	506	123.224
40	10	279	283	119	436	99.892
41	9	442	271	126	1686	479.919
42	9	297	300	136	498	125.536
43	8	293	289	152	424	103.372
44	5	278	349	83	423	151.728
45	5	335	383	155	469	134.659
46	5	325	396	143	423	125.949
47	5	332	356	138	494	158.665
48	5	380	410	237	517	124.357
49	5	306	360	174	427	108.544
50	4	380	433	202	452	120.120
51	4	521	414	277	981	313.882
52	3	371	440	213	460	137.179
53	3	283	213	196	440	136.090
54	3	369	320	253	533	146.219
55	3	399	488	172	537	198.064
56	3	401	465	164	573	212.089
57	3	442	566	162	598	242.864
58	2	275	275	65	486	297.924
59	2	430	430	172	689	365.437
60	2	380	380	151	608	323.031
61	2	451	451	183	719	379.060
62	2	483	483	174	792	436.963
Total	1053	275	221	0	2839	204.003

Table 4a: Serial Urinary neopterin levels during the systemic therapy in group A

Table 4b

Patient	Neopterin/Creatinine
1	170
2	180
3	183
4	208
5	352
6	437
7	485
8	571
9	617

Table 4b : Pre-treatment Neopterin in patients in whom serial sample collections could not be completed

The table 4a depicts the mean \pm standard deviation (SD) of interval between the treatment start and sample collection, number of patients examined, mean \pm SD of urinary neopterin at the respective visit.

Summary of results of part I

The mean (\pm standard deviation) of urinary neopterin at baseline was 272 ± 225 $\mu\text{mol/mol}$ Creatinine. A significant correlation was observed between urinary neopterin and peripheral blood leukocyte count ($r_s=0.38$; $p<0.05$; Figure 19), hemoglobin ($r_s= -0.34$; $p<0.05$; Figure 18) and CEA ($r_s=0.33$; $p<0.05$; Figure 20) concentrations. Seventeen patients had urinary neopterin ≥ 214 $\mu\text{mol/mol}$ creatinine.

Daily neopterin measurements were obtained from 36 patients (Figures 9-15). The mean number of measurements obtained was 21 ± 18 (range 1-58). Two fundamental patterns of urinary neopterin were evident based on initial neopterin concentrations. In patients with pre-treatment urinary neopterin ≥ 214 $\mu\text{mol/mol}$ creatinine, a stable or decreasing pattern of urinary neopterin concentrations was usually observed. In contrast, urinary neopterin increased significantly in patients with initial neopterin < 214 $\mu\text{mol/mol}$ creatinine (Figure 16, 17) . In the patient treated with single-agent cetuximab, an increase of urinary neopterin was observed despite elevated initial neopterin concentrations (Figure 15).

At the time of the analysis, 44 patients died while one patient was alive after 74 months. Survival of patients with urinary neopterin concentration of ≥ 214 $\mu\text{mol/mol}$ creatinine or above was significantly inferior compared to patients with initial urinary neopterin below 214 $\mu\text{mol/mol}$ creatinine (median 10.1 vs. 17.7 months, $p<0.05$; Figure 21).

6.2 Part II

6.2.1 Patient group - Cohort B

Patient group

The cohort B of patients was selected from the patients undergoing treatment for gynecological malignancies at the department of oncology and radiotherapy at University Hospital in Hradec Kralove.

Rationale for selection of cohort B

- a. Patients with carcinoma of cervix undergo a short course high intensity treatment that includes chemotherapy and radiotherapy.
- b. Cisplatin can cause significant toxicity and complication.
 - a. Significant doses of radiation are delivered in pelvic radiotherapy.
 - b. As a first step it would be sensible to monitor acute complications during the short course of intense treatment.
 - c. Since the patients spent majority of time in hospital during treatment, serial sample collection was logistically easier

Part II

Number of patients, n = 10

Diagnosis -

1. Patients with carcinoma of uterine cervix, n = 9
2. Patients with carcinoma of the vulva, n = 1

Table 5. The cohort of patients studied in cohort B. Age group, histology and staging

Patient	Age (years)	Histology	FIGO stage
1	50	Adenoc.	III.B
2	51	Adenoc.	II.B
3	60	Adenoc.	III.B
4	42	SCC	III.B
5	49	Adenoc.	II.B
6	36	SCC	III.B
7	70	Adenoc.	III.B
8	45	SCC	II.B
9	49	SCC	III.B
10	76	SCC (vulva)	Recurrent

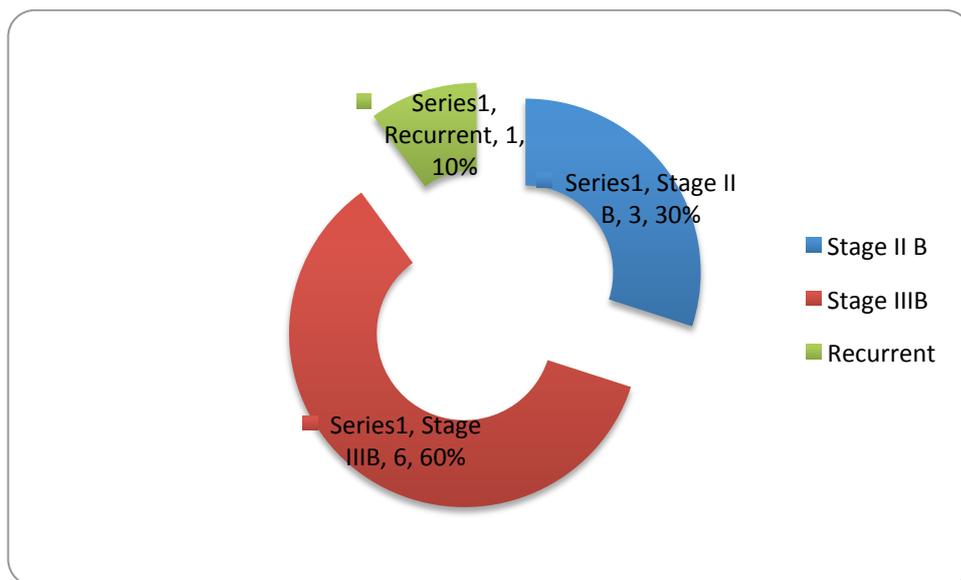


FIGURE 22 - Staging of patients in cohort B . The division of patients in cohort B based on staging is demonstrated in Figure 22

Treatment given to the patients in cohort B

1. Patients with carcinoma of the uterine cervix n= 9
Treatment – Underwent radiotherapy at doses of 50 Gy in 25 fractions.
All patients were treated with concomitant weekly Cisplatin (40mg/m²)
2. Patient with carcinoma of the vulva n=1
Treatment – Underwent radiotherapy alone

Summary of patient group B

Nine patients with carcinoma of the uterine cervix and one patient with carcinoma of the vulva treated with pelvic radiotherapy were included in the present analysis (Table 5).

Patients were staged according the Fédération Internationale de Gynécologie et d'Obstétrique (FIGO) classification [Quinn, 2006].

All patients with cervical carcinoma were treated with concomitant weekly cisplatin (40 mg/m²) while the patient treated for carcinoma of the vulva received radiotherapy alone.

Patients with carcinoma of the uterine cervix were treated with whole pelvis three-dimensional conformal radiotherapy using a linear accelerator with 18 MV photons. Dose was prescribed at the ICRU (International Commission on Radiation Units and Measurement) point and was 50 Gy in 25 fractions (2 Gy per fraction). In patients with cervical cancer, treated with intracavitary high dose rate brachytherapy, the dose was prescribed to a selected reference point “A” (defined as a point 2 cm lateral to the cervical canal and 2 cm superior to the ovoids.) Dose for organs at risk is reported using individual points for the bladder and rectum. Patients underwent 6 fractions of brachytherapy, 4 Gy per fraction, three fractions per week. The dose in the patient with recurrent carcinoma of the vulva was 50 Gy in 25 fractions to the vulva and bilateral inguinofemoral lymph nodes with the boost of 16 Gy in 8 fractions to the left groin.

6.2.2 Methodology

Methodology of sample collection and neopterin assessment has been described in Part 1 of the study

Samples collected for Cohort B

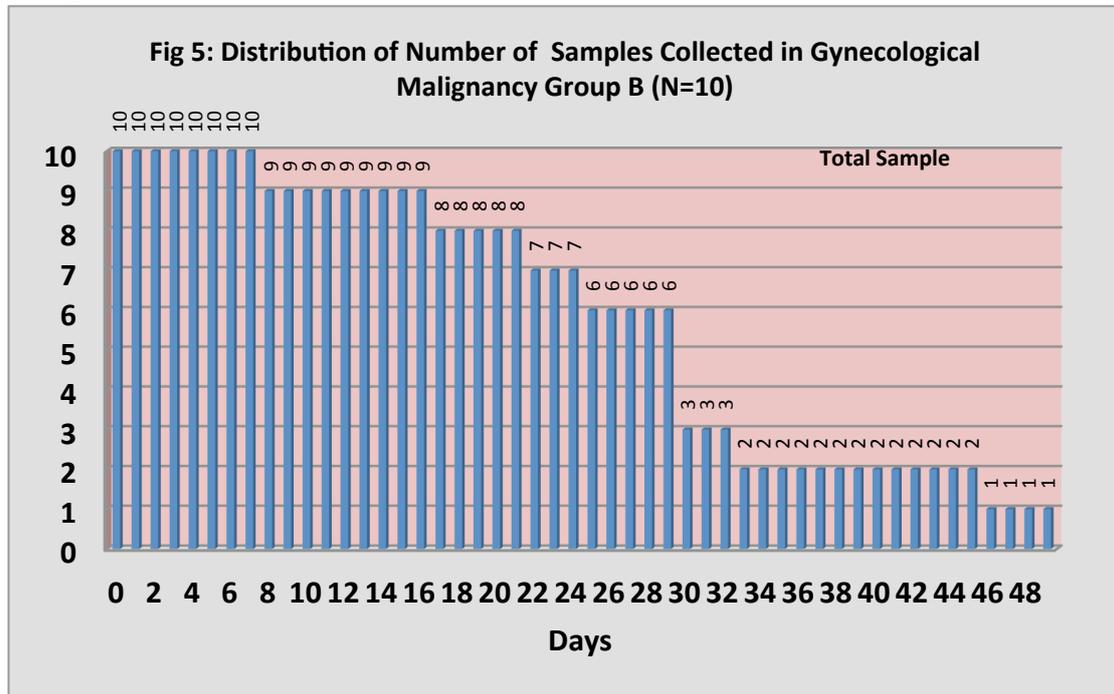


FIGURE 23- Number of samples analyzed the part 2 of the study on patients in cohort B. Number of daily samples analyzed the part 2 of the study on patients in cohort B is shown in figure 23

6.2.3 Statistics

Urinary neopterin concentrations before and during radiotherapy were compared using Wilcoxon signed rank test

The decision on statistical significance was based on $p = 0.05$ level.

The analyses were performed with NCSS software (Number Cruncher Statistical Systems, Kaysville, Utah, USA).

6.2.4 Results Table 6

Visit	Time (days post commencement of therapy)	n	Neopterin ($\mu\text{mol/mol}$ creatinine) (mean \pm SD)	Median	<i>p</i> -value
1		10	256 \pm 110	284.00	-
2	2 \pm 2	10	248 \pm 99	254.00	0.846
3	4 \pm 2	10	288 \pm 85	303.00	0.541
4	5 \pm 2	10	230 \pm 77	250.00	0.232
5	6 \pm 2	10	260 \pm 57	265.00	1.000
6	7 \pm 2	10	304 \pm 135	277.50	0.770
7	9 \pm 3	10	279 \pm 117	285.00	0.415
8	10 \pm 3	10	286 \pm 112	268.50	0.232
9	11 \pm 3	9	262 \pm 119	246.00	0.652
10	12 \pm 3	9	267 \pm 106	222.00	0.250
11	14 \pm 3	9	280 \pm 126	245.00	0.820
12	15 \pm 3	9	328 \pm 215	235.00	0.477
13	19 \pm 8	9	273 \pm 135	265.00	0.570
14	20 \pm 8	9	295 \pm 128	284.00	0.426
15	21 \pm 8	9	289 \pm 218	199.00	0.910
16	22 \pm 8	9	301 \pm 150	229.00	0.441
17	24 \pm 9	9	333 \pm 171	311.00	0.203
18	25 \pm 9	8	334 \pm 296	235.00	0.383
19	27 \pm 9	8	236 \pm 203	188.00	0.195
20	29 \pm 10	8	249 \pm 170	190.50	0.844
21	31 \pm 10	8	275 \pm 177	195.00	0.641
22	34 \pm 11	8	263 \pm 130	216.50	0.461
23	32 \pm 8	7	248 \pm 217	156.00	0.938
24	34 \pm 8	7	259 \pm 152	189.00	0.446
25	37 \pm 9	6	293 \pm 142	221.00	0.219

Table 6. Urinary neopterin in patients of cohort B during the course of pelvic radiation. Shown are the mean \pm standard deviation (SD) of interval between the treatment start and sample collection, number of patients examined, mean \pm SD of urinary neopterin and *p*-value (Wilcoxon signed rank test) at the respective visit.

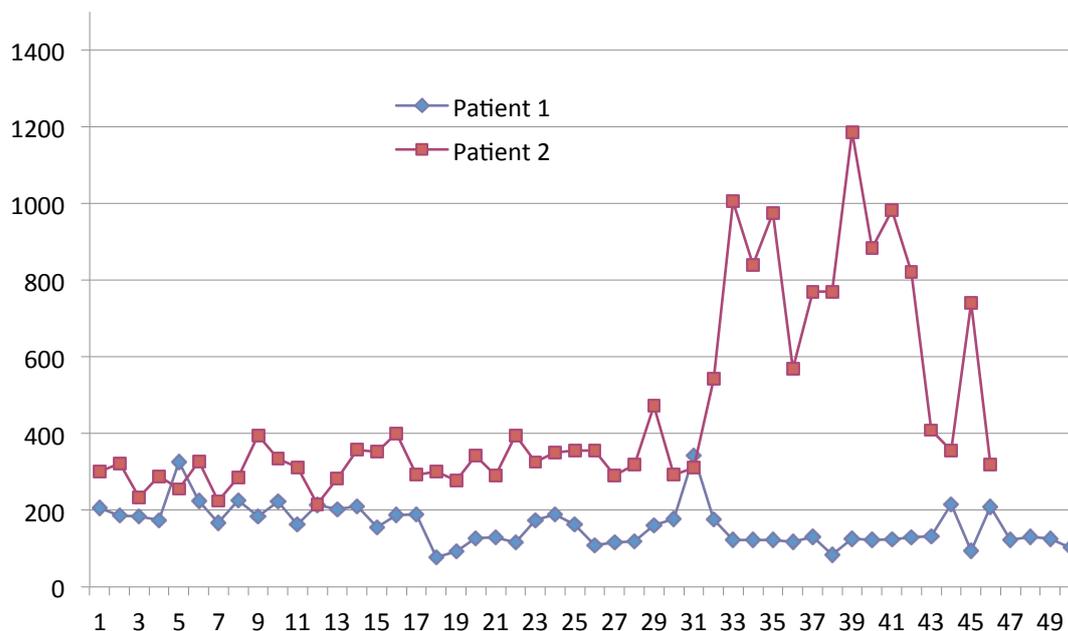


FIGURE 24 The course of urinary neopterin concentrations in patients 1 and 2 cohort B is shown.

Patient 1 had stage III B cervical carcinoma and baseline neopterin concentrations on the upper limits of the normal range (205 µmol/mol creatinine). External beam radiotherapy was started on the day of the visit 1 and administration of cisplatin was initiated on the day of the visit 4. Uterovaginal brachytherapy was started the day before the visit 35. Clinically, the course of treatment of this patient was uneventful. The therapy was complicated only by mild (grade 2) leukopenia. Only mild fluctuations of urinary neopterin concentrations were observed that were not accompanied by clinical symptoms.

Patient 2 who had stage III B cervical carcinoma and increased baseline urinary neopterin concentrations (301 µmol/mol creatinine) started external beam radiotherapy on the day of the visit 1 and the first administration of cisplatin on the day of the visit 6. Uterovaginal brachytherapy was initiated on the day of the visit 30, and continued for 6 fractions, with the last fraction administered on the days of the visit 39. On the day of the visit 37 the patient reported a burning sensation in the genital area. Systemic administration of ciprofloxacin and metronidazole was started and continued for 10 days. This episode coincided with a marked increase in urinary neopterin concentrations. Peak urinary neopterin level of 1187 µmol/mol creatinine was observed on the day of the visit 39 (on this day the patient reported chills).

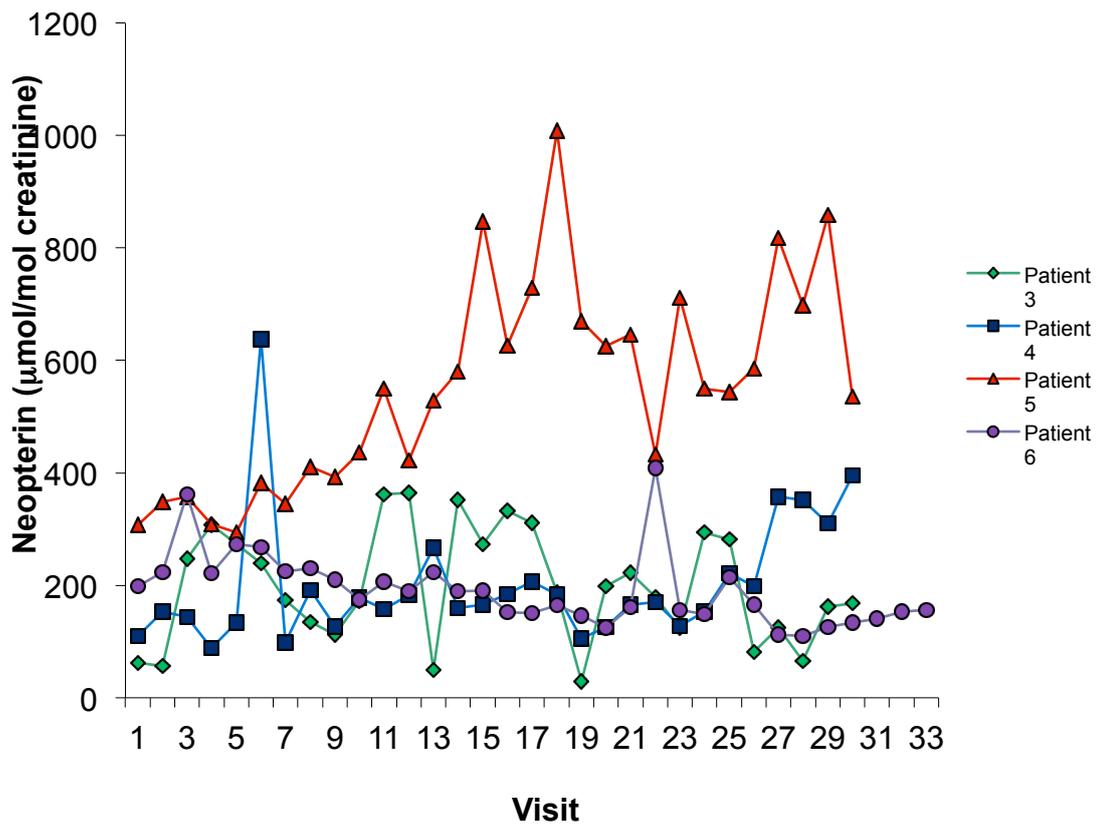


FIGURE 25. Urinary neopterin concentrations during the course of chemoradiation in patients 3 to 6 of cohort B

The figure shows the course of urinary neopterin during chemoradiation in 4 patients with cervical carcinoma. The marked increase of urinary neopterin concentrations in patient 5 starting with visit 11 was associated with dyspeptic complaints accompanied by diarrhea and fatigue. Subsequently, the patient had skin rash. The peaks of urinary neopterin concentrations in patients 3 and 4 were accompanied only by minor complaints.

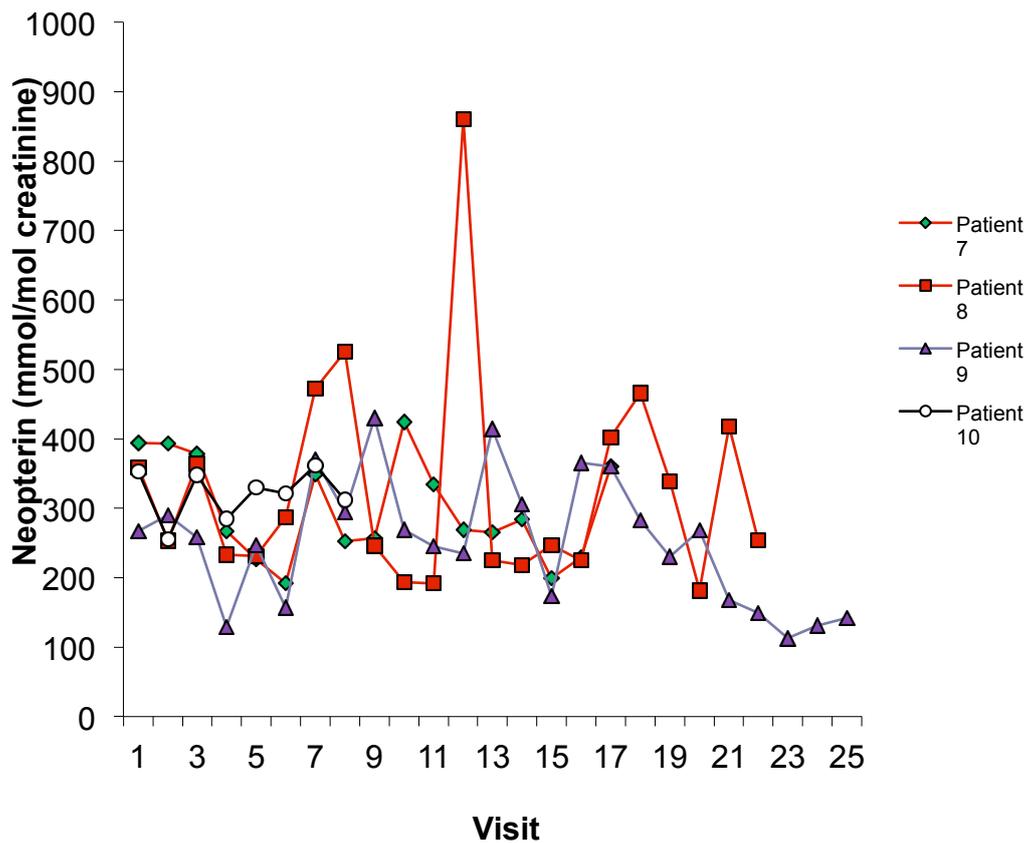


FIGURE 26. Urinary neopterin concentrations during the course of chemoradiation in patients 7 to 10 of cohort B

The figure shows the course of urinary neopterin during chemoradiation in 4 patients. In patient 8 the peak urinary concentration on the day of the visit 12 coincided with the manifestation of skin rash on lower extremities. Other peak neopterin values were accompanied only by minor complaints. While, patients 7, 8 and 9 had cervical carcinoma, patient 10 had recurrent carcinoma of the vulva and was followed only shortly because local reaction made the collection of urine specimens difficult.

Summary of results in cohort B

Baseline urinary neopterin concentrations were, generally, above the normal range. Urinary neopterin concentrations were relatively stable during the first five weeks of combined (chemo) radiation.

Marked peaks of neopterin concentrations reflected the emergence of complications. No statistically significant changes were observed when neopterin concentrations at each visit were compared to baseline using the Wilcoxon signed rank test.

In nine patients with cervical cancer treated with chemo radiation, no significant difference was observed between urinary neopterin concentrations before and at the end of treatment after (mean±Standard deviation) 47 ± 12 days (245 ± 111 vs 285 ± 121 $\mu\text{mol/mol}$ creatinine $P = 0.477$)

The Friedman test performed on the data of cervical cancer patients treated with chemo radiation also revealed no significant trend ($p = 0.861$).

7. Discussion

The study of the host immune response and immunomodulation promises to unravel new facets of pathogenesis of cancer and may pave way for new strategies for management of malignancy. Hence, biomarkers of immune activity will occupy an increasingly important role in modern oncology practice. However, in current clinical settings the use of biomarkers associated with the host response to neoplastic process is limited [Melichar 2013].

Neopterin is a well-established biomarker of immune system activation [Melichar et al 2006a, Wachter et al 1989] and prognostic significance of increased systemic neopterin concentrations has been demonstrated across a spectrum of malignant disorders [Reibneggar et al 1991]. This is based on the results of several studies in patients with tumors of variety of primary locations, including colorectal carcinoma, where increased serum or urinary neopterin concentrations were associated with poor prognosis [Melichar et al 2006a, Weiss et al 1993]. Our study was designed to test these findings and to establish the reproducibility of these results in clinical setting.

In addition, an anticancer therapy-induced rise of urinary neopterin concentrations has been described after the administration of chemotherapy or cytokines [Melichar et al 2006a, Melichar et al 2008a] and radiotherapy-associated changes have also been documented. Targeted therapy is a latest addition in the arsenal of oncologists. However, so far the information about neopterin in patients treated with targeted agents such as cetuximab is limited.

Our project was designed to test the clinical application of neopterin in management cancer patients.

Our data was able to demonstrate:

1. That the pretreatment neopterin correlated with prognosis of patients of metastatic colorectal carcinoma.
2. The differential behavior of urinary neopterin concentrations during anticancer therapy with the targeted agent (cetuximab) in combination with chemotherapy.
3. That rise or spikes in urinary neopterin concentration may indicate the presence of complications in patients undergoing anticancer treatment.

When treatment was initiated in our cohort of patients with colorectal cancer (cohort A) a significant increase in urinary neopterin was observed in patients with normal range of pretreatment concentration, while a decreasing trend was evident in patients with high initial urinary neopterin. These changes in neopterin levels underline the presence of systemic immune activation during the systemic therapy and use of cetuximab. Only one patient in our group was treated with cetuximab monotherapy, and hence it is difficult to discern the effects of irinotecan-based chemotherapy and administration of cetuximab.

The present study corroborates the observation of negative prognostic significance of increased urinary neopterin concentrations in patients with metastatic colorectal carcinoma [Melichar et al 2006c, Weiss et al 1993] and extends to patients treated in second or higher line of therapy with combination of chemotherapy and cetuximab. Whether the negative prognostic significance of high urinary neopterin concentrations observed in the present cohort is associated with the absence of systemic immune response, and if that is reflected by a lack of an increase of urinary neopterin concentrations, is a subject of further investigation.

Our study has corroborated the prior reports on correlation of neopterin concentrations with various laboratory parameters. We were able to identify a correlation between urinary neopterin, peripheral blood leukocyte count, hemoglobin, and CEA concentration. These correlations could partly explain the association between high urinary neopterin concentrations and poor prognosis in the patients of cohort A. The inverse correlation of hemoglobin with neopterin concentrations that has been studied extensively [Melichar et al 2008a, Fuchs et al 1991, Sramek et al 2013] has also been demonstrated in our cohort A. Previously, in a retrospective analysis of patients of advanced colorectal carcinoma neopterin along with CEA was found to be an indicator of prognosis [Melichar et al 2006c]. However, it was a retrospective analysis and our current prospective data corroborates these findings. In addition to the methodology, the major difference is the use of cetuximab in our present patient group. Again, 214 were the cut off between the two groups as in our present study. These cutoff limits were selected based on medians of respective parameters in the studied group.

Targeted anticancer therapy has been a promising development and has proved to be efficacious in not only extending lives but also in maintaining quality of life in

cancer patient. In spite of several studies, the precise the mechanism of action of many targeted agents is not entirely clear. In addition to targeting the intercellular pathways it may involve, at least in part, the activation of the immune response. The targeted agent that was administered to patients in our study was cetuximab.

Cetuximab is an IgG1 class antibody that could trigger antibody-dependent cell-mediated cytotoxicity [Messersmith et al 2007] and there is data indicating that, indeed, the activation of host response may be one of the mechanisms responsible for antitumor activity of cetuximab [Zhang et al 2007, Bibeau et al 2009]. The changes of urinary neopterin observed during the treatment in the present study further supports the notion that the activation of host response may represent one of the mechanisms behind anti-tumor activity of cetuximab alone or in combination with cytotoxic chemotherapy. A rise in the parameters of immune or inflammatory response may have different implications and reflect association with effective host response associated with tumor eradication.

On the other hand, increased neopterin concentrations before the start of therapy may indicate the presence of a state refractory to further stimulation of the immune system. In earlier studies, correlations were observed between lower numbers or impaired function of lymphocytes or dendritic cells and neopterin concentrations [Melichar et al 2001]. Thus, increased neopterin concentrations are thought to reflect immune dysregulation [Melichar et al 2006a].

There have been reports showing that when the daily monitoring of urinary neopterin was performed in cancer patients, an increase in neopterin concentrations preceded complications while a decrease in urinary neopterin was associated with tumor control [Melichar et al 2007]. Reports from studies based on patients with non-neoplastic disorders have been encouraging. For example daily neopterin measurements were reported in organ transplant recipients and a rise in urinary neopterin was an early indicator of acute complications [Chin et al 2008]. In oncology practice complications can also be due the direct side effects or adverse reactions to anticancer treatment. While some side effects of anticancer therapy, e.g., skin or eye toxicity [Melichar and Nemcova 2007], may be assessed directly by visual inspection, several adverse events of the treatment are not so easy to detect or evaluate. Anticancer therapies also make patients vulnerable to other complications such as infections. Neopterin may offer a tool to monitor such important but subtle complications.

As mentioned earlier some side effects might not be visible but can yet be clinically significant. Neopterin might be a useful to forewarn or monitor such complication. The best example of this potential is the successful implementation of neopterin for clinical practice has been in the area of transfusions.

In their communication to Lancet, Operskalski et al. highlighted the utility of neopterin in blood transfusion. They argued that there will be newer undiscovered viruses that would need to be screened prior to transfusions and due to its non-specificity but high sensitivity, neopterin is an excellent marker to screen acute infective phases in the donor blood. [Operskalski et al 1997]. This communication sums up the reason why Austria has used neopterin as a screening method in blood products since 1994. Screening of blood donations with neopterin allowed the detection and exclusion of viral infections during the acute phase when virus load is highest and allows to further shortening the diagnostic window in addition to specific serologic screening methods [Zangerle et al 1992, Reissigl et al 1989]. In this way subclinical infections or silent systemic disorders may be detected in a higher frequency by increased neopterin concentrations and suspicious blood units are discarded to increase the security of transfusion [Hönlinger et al 1989].

In case of management of cancer patients the complications related or unrelated to therapy could, depending on their grade or extent, have a huge impact on the overall outcome of treatment. A practicing oncologist is always weary of such a situation. It is not uncommon for a patient to miss chemotherapy or other treatment schedule because of certain complications. An observation of routine oncology practice would reveal that complications might lead to the following.

Unwanted delays – Example - mucositis related to a drug or radiotherapy affect the patients swallowing and hence the oral treatment cannot be continued; it also leads to nutritional deficiency leading to morbidity.

Dose reduction - Example - Neuropathy can have an adverse impact on the performance status, thereby mandating reduction in the dose.

Hospital admissions - Example - Myelosuppression can lead to anemia or bleeding and require transfusion.

Mortality – Example - Neutropenic sepsis, major bleeds again a potential

complication of cytotoxic therapy. Some drugs can cause anaphylactic reactions too.

All the above can derail the treatment plan either in part or in its entirety. Hence, a tool for objective assessment of complication is highly desirable in clinical practice. Neopterin has been associated with side effects and complications in previous studies and our study corroborates the findings. With further to data support our findings, the measurement of neopterin in the urine could offer a non-invasive approach for the assessment of the condition of the patient, and it could be of special value in the outpatient setting.

In the second part of our study we monitored serial urinary neopterin levels in patients of gynecological malignancy who were undergoing pelvic radiotherapy with concomitant chemotherapy (cohort B). Pelvic chemoradiation is an effective therapeutic modality in adjuvant treatment as well as in patients with inoperable cervical carcinoma [Morris et al 1999, Keys et al 1999]. However, chemoradiation is an aggressive therapy that results in a significant percentage of serious, in extreme cases even lethal, complications. Timely management of complications of therapy is of great importance.

In our study, we were unable to detect any significant increase in urinary neopterin concentrations during external beam radiation in patients with gynecological cancer. As explained in the previous section, all but one of our patients had cervical carcinoma. Our data indicated that, in the absence of complications, urinary neopterin concentrations show only mild fluctuation throughout the course of therapy without a significant trend.

These negative findings contrast with our data from the cohort A where we found definitive change in urinary neopterin levels in response to the anticancer therapy and also with the recently reported in a cohort of patients with head and neck carcinoma [Holeckova et al 2012, Holecková et al 2013]. In fact, it might be expected that the chemoradiation regimen used in cervical cancer would result in a marked activation of systemic immune response reflected in increased neopterin concentrations. However, our results did not qualify the above statement which is based on the fact that all major cell populations responsible for the host response to neoplasia are present in the peritoneal cavity, including monocytes/macrophages [Melichar and Freedman 2002] and these cells may be activated by therapeutic

manipulations [Freedman et al 2003]. Moreover, both chemotherapy and radiation cause a significant damage to the intestinal barrier [Dvorak et al 2010] that may result in the activation of the systemic immune response. This could not be observed in cohort B. However, in individual patients of cohort B, a marked increase (spikes) in urinary neopterin concentration was noted. These spikes in neopterin levels coincided with clinically demonstrable complications. The fact that the neopterin failed to change in response to treatment provided a backdrop plateau to observe the spikes in urinary neopterin levels. Hence, we can say that in cohort B the rise in neopterin reflected the emergence of the complications during therapy rather than a direct effect of the treatment itself. True to the non-specific character of neopterin, the rise did not accompany any specific condition but were associated with different complications. Similar to the results of the present study, no significant increase in urinary neopterin concentrations was reported earlier in patients with rectal cancer treated with chemoradiation [Dvorak et al 2010]. Why these groups behaved differently is a matter of further investigation.

In our study, the pretreatment urinary neopterin concentrations were relatively high and above normal range in most patients. High neopterin concentrations in cancer patients may be associated with a down-regulation of immune response [Melichar et al 1996, Melichar et al 2001] and the immune system plays an important role in the progression of abdominal and pelvic neoplasms [Melichar et al 2002]. High neopterin concentrations may decrease as a consequence of tumor control. Increased urinary neopterin concentration is also an independent parameter associated with poor prognosis in cervical cancer [Reibnegger et al 1996]. However, in the present study, the number of patients examined was too small to analyze an association between neopterin concentrations before or during chemoradiation with the outcome.

The fact that neopterin concentrations may also increase as a result of non-neoplastic disorders represents an advantage and assessment of neopterin levels may help to detect or monitor a wide range of different complications that could impact the effectiveness anticancer therapy. Neopterin levels do reflect immune activation in cancer patients however it lacks the specificity required to formulate a strategy in a cancer subtypes. However, several problems associated with different malignancy and with different anticancer treatment are common and even their manifestations are non-specific. The best example is the commonality of side effects of cytotoxic

chemotherapy and of targeted therapy too.

There are several possible explanations for the negative findings in the present study with cohort B. Our cohort, comprising of 10 patients, might be relatively small group to predict general trend. The samples in our study were usually not collected during treatment interruptions, making comparison of values obtained at the same visit in different patients difficult. Consequently, statistical analyses performed here have to be regarded as exploratory at best. Future investigations on a larger cohort of patients to investigate the potential of neopterin as a biomarker for early detection of complications during pelvic radiotherapy as well as other anticancer treatment ought to be undertaken.

The association of the changes in urinary neopterin concentrations during the treatment with biological therapy to survival could be investigated in other cancer type and with other agents. It might also give further insights to the mechanism of action of targeted therapy and its interaction with systemic immune activity.

In light of our results we believe that neopterin is a promising biomarker that serves not only as a tool for laboratory based investigations and research but that it also has a potential to be of assistance in the clinical management of several aspects in oncology practice. This includes prognosticating, monitoring complications and response to anticancer therapy.

8. Conclusion

Host immune activity plays a vital role in the pathogenesis, prognosis and response to treatment in cancer patients. Neopterin, a laboratory biomarker of immune activation, was tested for its potential use in clinical setting during the management of cancer patients.

Our study was focused on three main aspects namely prognosis, monitoring response to therapy and monitoring complications.

We found that in cohort A, a higher neopterin levels were associated with poor prognosis. Neopterin levels in patients with higher initial levels, fell while on treatment. In this cohort neopterin levels showed correlation to hemoglobin levels, white cell count and CEA. Based on the results of the first part we could possibly conclude that urinary neopterin could be prognostic biomarker in patients treated with systemic therapy in second or higher line of treatment for metastatic colorectal cancer.

Interestingly, traditional anticancer therapy and even the newer targeted therapy are proving to have immunomodulatory effects contributing to their anticancer activity. We were able to monitor the fluctuations and trends of change in neopterin levels in cohort A, whilst on treatment. A marked increase of urinary neopterin observed during the treatment may indicate an activation of immune response.

In cohort B, pretreatment neopterin levels were generally higher. Contrary to our expectation, in this part of the study we were unable to detect any significant change in urinary neopterin concentration in patients treated with pelvic (chemo) radiation. However, we were able to demonstrate that urinary neopterin concentrations may reflect the complications during therapy and could be used to monitor the condition of the patient during the treatment.

The advantage being that a spiking neopterin level could alert a vigilant physician who can anticipate complications or ensure that adverse events are picked up in time.

In our study we were able to recruit patient of different kinds of malignancies. We monitored neopterin in patients while undergoing all established non-surgical anticancer therapy including chemotherapy, radiotherapy and targeted therapy. We

looked as various aspects like, prognosis, monitoring response to therapy and monitoring complication in cancer patients.

The ability of measuring neopterin in urine gives it a unique edge over other biomarker for example, CRP, ferritin and albumin, all of which require phlebotomy.

Taking into consideration patient discomfort, problems like thrombophlebitis due to repeated venipuncture, the requirement of trained phlebotomy personal, the risk of contamination and sharps injury, one could argue that measurement of a urinary biomarker is more patient friendly and cost effective.

It must be mentioned that this is probably a unique study in terms of monitoring neopterin levels during cetuximab infusion in this patient subset. Furthermore, the findings from this study have also added to the current body of work on neopterin in form of publications and it not only corroborates previous findings but also contributes to the knowledge. With the evidence of therapy related changes in neopterin levels the current findings also adds to a growing body of literature establishing immune mechanisms of targeted agents.

The intention of our project was to test the hypothesis that neopterin has the potential to be of use in routine oncology practice. We believe that we have been able to confirm that neopterin has a potential role in prognosticating and monitoring response to therapy and complications in patients undergoing anticancer therapy.

In view of our result it would be fair to say that neopterin has the potential to walk out of the realms of laboratory research into the domain of routine clinical activity in modern oncology practice. It must be added that much work with stronger evidence will be needed to sieve out clinical applications of neopterin in the clinics. Our study on the specific issues of assessment of prognosis, monitoring response to therapy and complications in cancer patients may provide a building block in this exercise.

9. Limitations of the current study

Although the study has successfully demonstrated that neopterin can potentially play a role in clinical settings, our study did have certain limitations.

The generalizability of these results is subject to certain limitations. For instance, there is a difference in the percentages of patients with different malignant pathologies in terms of rise of neopterin. In our study we had recruited patient with two different common types of cancer, however in order to recommend the use of neopterin in routine oncology practice similar prospective studies in other patient groups ought to undertaken.

In addition, our study was limited with the number of patients and sample size. It was possibly because of this drawback that we were unable to demonstrate therapy related change or a trend in cohort B. Hence the results in second cohort may be regarded as exploratory at best. Our study was based in a single center and the number of patients qualifying for study can be limited. Hence, a larger multi-centric study with neopterin to address specific question could be designed.

Secondly, we were not able to collect sample from patients during bank holidays and weekend in the second part of our study. We could have missed small fluctuations in neopterin levels during this period. Further research may shed light on why certain patients undergoing chemoradiotherapy do not show changes in neopterin levels.

Further refinements and larger studies to investigate specific use of neopterin would be needed to corroborate the routine use of neopterin in clinical practice.

10. Recommendations

Our understanding of immune system, its role in cancer development and the immune escape mechanism of malignant cells is evolving. Neopterin with its characteristic property of reflecting the immune activation provides an essential tool in further research. The role of neopterin and how it affects the microscopic and macroscopic homeostatic mechanism also needs to be looked at. In addition, with further expansion of clinical studies and collecting evidence of the correlation of neopterin with various laboratory and clinical parameters we might be able to soon see this long lost compound on the panel of investigations that oncologist request while managing specific patients.

Our research has also thrown up questions that need to be answered by further investigations. It will be interesting to see if a larger study on patients with pelvic chemoradiation would yield a different result. A multi-centric study with specific subdivision based on tumor types and stages and treatment modality could be undertaken to expound on the current results.

It will also be very interesting to observe the effect of new immunomodulatory anticancer therapy on neopterin levels. The urinary neopterin levels in responders and non-responders to these drugs could be a good initial starting point.

Our study was also able to demonstrate that neopterin could be a potential tool in better understating of the effects of targeted therapy on the immune system. This could be potentially used for other studies to understand the mechanism of action of targeted therapy and to tailor the treatment in the new age of personalized medicine.

11. References

- Abate G, Comella P, Marfella A, Santelli G, Nitsch F, Fiore M, et al. Prognostic relevance of urinary neopterin in non-Hodgkin's lymphomas. *Cancer* 1989;63(3):484–9
- Andert SE, Greismacher A, Zuckermann A, Muller MM. Neopterin release from human endothelial cells is triggered by interferon-gamma. *Clin Exp Immunol* 1992;88:555–8.
- Baier-Bitterlich G, Fuchs D, Murr C et al. Effect of neopterin and 7, 8–dihydroneopterin on tumor necrosis factor- α induced programmed cell death. *FEBS Lett* 1995;364:234–8.
- Balkwill F, Mantovani A. Inflammation and cancer: back to Virchow? *Lancet* 2001;357:539–45.
- Bayram M, Bayram O, Boyunaga H, Ozer G, A research on the level of urine neopterin to see if it may provide a vital clue for a provisional diagnosis of breast cancer in menopausal women, *Maturitas* 48 (2004) 432–437.
- Becker K, Geisler S, Ueberall F, Fuchs D and Gostner J, Immunomodulatory properties of cacao extracts – potential consequences for medical applications. *Front. Pharmacol.*, 12 December 2013
- Berdowska A, Zwirska-Korczala K, Neopterin measurement in clinical diagnosis, *J. Clin.Pharm.Ther.*26(2001)319 – 329.
- Bibeau F, Lopez-Crapez E, Di Fiore F, Thezenas S, Ychou M, Blanchard F, Lamy A, Penault-Llorca F, Frebourg T, Michel P, Sabourin J-C and Boissiere-Michot F. Impact of Fc γ RIIa-Fc γ RIIIa polymorphisms and KRAS mutations on the clinical outcome of patients with metastatic colorectal cancer treated with cetuximab plus irinotecan. *J Clin Oncol* 27: 1122-1129, 2009.

Bokemeyer C, Bondarenko I, Makhson A, Hartmann JT, Aparicio J, de Braud F, Donea S, Ludwig H, Schuch G, Stroh G, Loos AH, Zube A and Koralewski P: Fluorouracil, leucovorin, and oxaliplatin with and without cetuximab in the first-line treatment of metastatic colorectal cancer. *J Clin Oncol* 27: 663-671, 2009.

Bokemeyer C, Van Cutsem E, Rougier P, Ciardiello F, Heeger S, Schliching M, Celik I, Köhne CH. Addition of cetuximab to chemotherapy as first-line treatment for KRAS wild-type metastatic colorectal cancer: pooled analysis of the CRYSTAL and OPUS randomised clinical trials. *Eur J Cancer*. 2012 Jul;48(10):1466-75.

Boon T, Van der Bruggen P, Human tumor antigens recognized by T-lymphocytes. *J Exp. Med.* 183 (1996) 725–729.

Bracci L, Schiavoni G, Sistigu A, Belardelli F, Immune-based mechanisms of cytotoxic chemotherapy: implications for the design of novel and rationale-based combined treatments against cancer *Cell Death and Differentiation* (2014) 21, 15–25

Bryniarski K, Szczepanik M, Ptak M, Zemelka M, Ptak W. Influence of cyclophosphamide and its metabolic products on the activity of peritoneal macrophages in mice. *Pharmacol Rep.* 2009 May-Jun;61(3):550-7.

Buttiglieri S, Galetto A, Forno S, De Andrea M, Matera L. Influence of drug-induced apoptotic death on processing and presentation of tumor antigens by dendritic cells. *Int J Cancer* 2003; 106: 516–520.

Capuron L, Geisler S, Kurz K, Leblhuber F, Sperner-Unterwieser B, Fuchs D. Activated Immune System and Inflammation in Healthy Ageing: Relevance for Tryptophan and Neopterin Metabolism. *Curr Pharm Des.* 2014 Mar 17. [abstract]

Carey B, Jain R, Adams, Wong K, Shaw S, Tse W, Kaminski E, Serum neopterin as an indicator of increased risk of renal allograft rejection. *Transplant Immunology Volume 28, Issues 2–3, March 2013, Pages 81–85*

Cesur S. Neopterin: a marker used for monitoring infections. *Mikrobiyol Bul.* 2005

Apr;39(2):251-60.

Chin GK, Adams CL, Carey BS, Shaw S, Tse WY, Kaminski ER. The value of serum neopterin, interferon-gamma levels and interleukin-12B polymorphisms in predicting acute renal allograft rejection. *Clin Exp Immunol.* 2008 May;152(2):239-44.

Colotta F, Allavena P, Sica A, Garlanda C, Mantovani A. Cancer-related inflammation, the seventh hallmark of cancer: links to genetic instability. *Carcinogenesis.* 2009 Jul;30(7):1073-81

Conrad F, Fuchs D, Haussen A, Salzer GM, Reibnegger G, Wachter H. Prognostic value of neopterin in patients with lung cancer. In: Pfeleider W, Wachter H, Blair JA, editors. *Biochemical and clinical aspects of pteridines.* Berlin: Walter de Gruyter; 1987. p. 233–41.

Correale P , Cusi M, Tagliaferri P. Immunomodulatory properties of anticancer monoclonal antibodies: is the ‘magic bullet’ still a reliable paradigm? *Immunotherapy* (2011) 3(1), 1–4

Denz H, Huber P, Landmann R, Wachter H, Fuchs D, Association between the activation of macrophages changes of iron metabolism and the degree of anaemia in patients with malignant disorders, *Eur. J. Haematol.* 48 (1992) 244–248

Dranoff G. & Mulligan, R. C. Gene transfer as cancer therapy. *Adv. Immunol.* 58, 417–454 (1995).

Dranoff G . Cytokine in cancer pathogenesis and cancer therapy. *Nature Reviews Volume 4;Jan 2004 ;11*

Dvorák J, Melichar B, Hyspler R, Krcmová L, Urbánek L, Kalábová H, Kasparová M, Solichová D. Intestinal permeability, vitamin A absorption, alpha-tocopherol, and neopterin in patients with rectal carcinoma treated with chemoradiation. *Med Oncol.* 2010 Sep;27(3):690-6.

Flavall, Crone E, Moore G, Giese S. Dissociation of neopterin and 78-dihydroneopterin from plasma components before HPLC analysis, *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* 863 (2008) 167–171.

Freedman RS, Vadhan-Raj S, Butts C, Savary C, Melichar B, Verschraegen C, et al. Pilot study of Flt3 ligand comparing intraperitoneal with subcutaneous routes on hematologic and immunologic responses in patients with peritoneal carcinomatosis and mesotheliomas. *Clin Cancer Res* 2003;9:5228–37.

Fuchs D, Hausen A, Huber C, Reibnegger G, Wachter H. Urinary neopterin in the diagnosis and follow-up of neoplasia: a biochemical parameter to detect cell-mediated immune response. *Tumour Biol.* 1984;5(3-4):199-209.

Fuchs D, Hausen A, Reibnegger G et al. Neopterin as a marker for activated cell mediated immunity: application in HIV infection. *Immunol Today* 1988;9:150–5 a

Fuchs D, Hausen A, Reibnegger G, Werner ER, Dittrich P, Wachter H. Neopterin levels in long term hemodialysis. *Clin Nephrol* 1988;177:1-6. b

Fuchs D, Milstien S, Kramer A et al. Urinary neopterin concentrations vs total neopterin for clinical utility. *Clin Chem* 1989;35:2305–7.

Fuchs D, Hausen A, Reibnegger G, Werner ER, Werner-Felmayer G, Dierich M and Wachter H: Immune activation and the anaemia associated with chronic inflammatory disorders. *Eur J Haematol* 46: 65-70, 1991.

Fuchs D, Murr C, Reibnegger G, Weiss G, Werner E, Werner-Felmayer G, Wachter H. Nitric oxide synthase and antimicrobial armature of human macrophages, *J. Infect. Dis.* 169 (1994) 224. a

Fuchs D, Stahl-Hennig C, Gruber A, Murr C, Hunsmann G, Wachter H, Neopterin—its clinical use in urinalysis, *Kydney Int* 1994;46:8-11 b

Fuith L, Fuchs D, Reibnegger G, Wachter H, Cellular immune activation and erythropoiesis in gynaecological cancer, *Lancet* 1 (1989) 908.

Ghiringhelli F, Menard C, Puig PE, Ladoire S, Roux S, Martin F et al. Metronomic cyclophosphamide regimen selectively depletes CD4⁺ CD25⁺ regulatory T-cells and restores T and NK effector functions in end stage cancer patients. *Cancer Immunol Immunother* 2007; 56: 641–648.

Giese S, Maghzal G, Glubb D. Protection of erythrocytes by the macrophage synthesized antioxidant 7,8 dihydroneopterin. *Free Radical Res.* 34 (2001) 123–136.

Gorin JB, Ménager J, Gouard S, Maurel C, Guilloux Y, Faivre-Chauvet A, Morgenstern A, Bruchertseifer F, Chérel M, Davodeau F, Gaschet J. Antitumor Immunity Induced after α Irradiation. *Neoplasia.* 2014 Apr;16(4):319-28.

Hagberg L, Cinque P, Gisslen M, Brew BJ, Spudich S, Bestetti A, Price RW, Fuchs D - Cerebrospinal fluid neopterin: an informative biomarker of central nervous system immune activation in HIV-1 infection. *AIDS Res Ther.* 2010 Jun 3;7:15.

Haberkorn J, Burbaum C, Fritzsche K, Geser W et al, Day-to-day cause effect relations between cellular immune activity, fatigue and mood in a patient with prior breast cancer and current cancer-related fatigue and depression. *Psychoneuroendocrinology* (2013) 38, 2366—2372

Hagemann T, Balkwill F, Lawrence T. Inflammation and Cancer: A Double-Edged Sword. *Cancer Cell.* 2007 October ; 12(4): 300–301

Hanahan, D. & Weinberg, R. A. The hallmarks of cancer. *Cell* 100, 57–70 (2000).

Handan Akbuluta, İlhami Celikb, Ayhan Akbulutb et al Serum neopterin levels in patients with brucellosis. *Journal of Infection* (2005) 51, 281–286

Hausen A, Fuchs D, Grunewald K, Huber H, Koenig K, Wachter H, Urinary neopterin as marker for haematological neoplasias, *Clin. Chim. Acta* 117 (1981) 297–305.

Holecková P, Krcmová L, Kalábová H, Kašparová M, Plíšek J, Pála M, et al. Prognostic significance of serum retinol, serum alpha-tocopherol, and urinary neopterin in patients with head and neck carcinoma treated with external beam radiation. *Int J Vitamin Nutr Res* 2012;82:77–84.

Holečková P, Krcmová L, Létal J, Svobodník A, Kalábová H, Kašparová M, et al. Urinary neopterin concentration and toxicity of radiotherapy in patients with head and neck carcinoma during external beam radiation. *Anticancer Res* 2013;33:4097–101.

Hönlinger, M.; Fuchs, D.; Hausen, A.; Reibnegger, G.; Schönitzer, D.; Werner, E.R.; Reissigl, H.; Dierich, M.P. and Wachter, H. (1989) *Dtsch. Med. Wochenschr.*, 114, 172-176. (Abstract)

Hoffmann B, Bass H, Nishanian P et al. Different lymphoid cell populations produce varied levels of neopterin, beta2-micro-globulin and soluble IL-2 receptor when stimulated with IL-2, interferon-gamma or tumor necrosis factor-alpha. *Clin Exp Immunol* 1992;88:548–54.

Hoffmann G, Wirleitner B, Fuchs D. Potential role of immune system activation associated production of neopterin derivatives in humans. *Inflammation Res.* 52 (2003) 313–321.

Hsu Y, Ajona D, Corrales L, Lopez-Picazo J, Gurrpide A, Montuenga L and Pio R. Complement activation mediates cetuximab inhibition of non-small cell lung cancer tumor growth in vivo. *Molecular Cancer* 2010, 9:139

Javeed A, Ashraf M, Riaz A, Ghafoor A, Afzal S, Mukhtar MM. Paclitaxel and immune system. *Eur J Pharm Sci* 2009; 38: 283–290.

Ji JH, Shin DG, Kwon Y, Cho DH, Lee KB, Park SS, et al. Clinical correlation between gastric cancer type and serum selenium and zinc levels. *J Gastric Cancer* 2012;12:217-22.

Ikeda H, Old L, Schreiber R. The roles of IFN gamma in protection against tumor development and cancer immunoediting. *Cytokine Growth Factor Rev.* 13 (2002) 95–109.

Inui A. Cancer anorexia-cachexia syndrome: are neuropeptides the key, *Cancer Res.* 59 (1999) 4493–4501

Kaneno R, Shurin G, Tourkova I, Shurin M. Chemomodulation of human dendritic cell function by antineoplastic agents in low noncytotoxic concentrations. *J Transl Med* 2009; 7: 58.

Keys HM, Bundy BN, Stehman FB, Muderspach LI, Chafe WE, Suggs CL 3rd, et al. Cisplatin, radiation, and adjuvant hysterectomy compared with radiation and adjuvant hysterectomy for bulky stage IB cervical carcinoma. *N Engl J Med* 1999;340:1154–61.

Khallouf H, Marten A, Serba S, Teichgraber V, Buchler MW, Jager Detal. 5-Fluorouracil and interferon-alpha immunochemotherapy enhances immunogenicity of murine pancreatic cancer through upregulation of NKG2D ligands and MHC class I. *J Immunother* 2012; 35: 245–253

Kurzrock R, The role of cytokines in cancer-related fatigue, *Cancer* 92 (2001) 1684–1688

Laich A, Neurauter G, Wirleitner B, Fuchs D. Degradation of serum neopterin during daylight exposure, *Clin. Chim. Acta.* 322 (2002) 175–178.

Laird BJ, Scott AC, Colvin LA, McKeon AL, Murray GD, Fearon KC, Fallon MT. Cancer pain and its relationship to systemic inflammation: an exploratory study. *Pain*. 2011 Feb;152(2):460-3

Leitner KL, Meyer M, Leimbacher W et al. Low tetrahydrobiopterin biosynthetic capacity of human monocytes is caused by exon skipping in 6-pyruvoyl tetrahydropterin synthase. *Biochem J* 2003; 373:681–8.

Ogawa M, Yu W, Iindaira K, Iwasaki M et al. Multiple Roles of Interferon- γ in the mediation of Interleukin 12-induced Tumor Regression. *Cancer Research* 58. 2426-2432. June 1. 1998

Mayersbach P, Augustin R, Schennach H, Schoenitzer D, Werner E, Wachter H, Reibnegger G. Commercial enzyme-linked immunosorbent assay for neopterin detection in blood donations compared with RIA and HPLC, *Clin. Chem.* 40 (1994) 265–266.

Melichar B, Jandik P, Krejsek J, Solichova D, Drahosova M, Skopec F, et al. Mitogen-induced lymphocyte proliferation and systemic immune activation in cancer patients. *Tumori* 1996;82:218–20.

Melichar B, Savary C, Kudelka A, Verschraegen C, Kavanagh J, Edwards C, Platsoucas C, Freedman R. Lineage-negative human leukocyte antigen-DR+ cells with the phenotype of undifferentiated dendritic cells in patients with carcinoma of the abdomen and pelvis. *Clin. Cancer Res.* 4 (1998) 799–809

Melichar B, Touskova M, Solichova D, Kralickova P, Kopecky O. CD4+ T-lymphocytopenia and systemic immune activation in patients with primary and secondary liver tumours. *Scand J Clin Lab Inv* 2001;61:363–70.

Melichar B, Freedman RS. Immunology of the peritoneal cavity: Relevance for host-tumor relation. *Int J Gynecol Cancer* 2002;12:3–17.

Melichar B, Slochova D, Freedman R. Neopterin as an indicator of immune activation and prognosis in patients with gynecological malignancies *Int J Gynecol Cancer* 2006, 16, 240–252 a

Melichar B, Solichova D, Melicharova K, Cermanova M, Urminska H and Ryska A. Systemic immune activation, anemia and thrombocytosis in breast cancer patients treated by doxorubicin and paclitaxel. *Pteridines* 17: 107-114, 2006 b

Melichar B, Solichova D, Melicharova K, Malirova E, Cermanova M and Zadak Z. Urinary neopterin in patients with advanced colorectal carcinoma. *Int J Biol Markers* 21: 190-198, 2006. c

Melichar B, Kalabova H, Urbanek L, Malirova E and Solichova D. Serial urinary neopterin measurements reflect the disease course in patients with epithelial ovarian carcinoma treated with paclitaxel/platinum. *Pteridines* 18: 1-7, 2007 a

Melichar B, Nemcová I. Eye complications of cetuximab therapy. *Eur J Cancer Care* 2007;16:439–43 (2007 b)

Melichar B, Urbanek L, Krcmova L, Kalabova H, Melicharova K, Malirova E, Hornychova H, Ryska A, Hyspler R and Solichova D. Urinary neopterin, hemoglobin and peripheral blood cell counts in breast carcinoma patients treated with dose-dense chemotherapy. *Anticancer Res* 28: 2389-2396, 2008. a

Melichar B, Dvorak J, Krcmova J et al. Intestinal permeability and vitamin A absorption in patients with chemotherapy-induced diarrhea. *Am J Clin Oncol* 2008 Dec;31(6):580-4. b

Melichar B. Laboratory medicine and medical oncology: the tale of two Cinderellas. *Clin Chem Lab Med* 51: 99-112, 2013.

Melicharová K, Kalábová H, Krcmová L, Urbánek L, Solichová D, Melichar B. Effect of comorbidity on urinary neopterin in patients with breast carcinoma. *Eur J Cancer Care (Engl)*. 2010 May;19(3):340-5.

Messersmith WA and Hidalgo M: Panitumumab, a monoclonal anti-epidermal growth factor receptor antibody in colorectal cancer: another one or the one? *Clin Cancer Res* 13: 4664-4666, 2007.

Moldawer L, Copeland E. Proinflammatory cytokines, nutritional support, and the cachexia syndrome: interactions and therapeutic options, *Cancer* 79 (1997) 1828–1839.

Mommsen P, Frink M, Pape H, Van Griensven M, Probst C, Gaulke R, Krettek C, Hildebrand F. Elevated systemic IL-18 and neopterin levels are associated with posttraumatic complications among patients with multiple injuries: A prospective cohort study. *Injury, Injury, Volume 40, Issue 5, May 2009, Pages 528-534*

Morris M, Eifel PJ, Lu J, Grigsby PW, Levenback C, Stevens RE, et al. Pelvic radiation with concurrent chemotherapy compared with pelvic and para-aortic radiation for high-risk cervical cancer. *N Engl J Med* 1999;340:1137–43.

Mountabarrik A, Takahara S, Nakanishi I et al. Interferon-gamma stimulates neopterin release from cultured kidney epithelial cells. *Scand J Immunol* 1994;39:27–30

Murr C, Neurauter G, Wirleitner B, Fuchs D. Neopterin to predict prognosis of infection and neoplasia, *International EUROGIN-EAST conference Vilnius, 8 - 10 september 2001 Link - <http://www.tmi.vu.lt/legacy/eurogin-east/neopterin.htm>*

Murr C, Widner B, Wirleitner B, Fuchs D. Neopterin as a marker for immune system activation. *Curr Drug Metab*. 2002 Apr;3(2):175-87

Neurauter G, Schroecksnadel K, Scholl-Bürgi S, Sperner- Unterweger B, Schubert C, Ledochowski M, Fuchs D. Chronic immune stimulation correlates with reduced phenylalanine turnover. *Curr. Drug Metab.* 9 (2008) 622–627.

Operskalski E, Busch M, Mosley J, Kedes D. Blood donations and viruses; *Lancet Vol* 349 , 1327 May 3, 1997

Oxenkrug G, Tucker KL, Requintina P, Summergrad P. Neopterin, a Marker of Interferon-Gamma-Inducible Inflammation, Correlates with Pyridoxal-5'-Phosphate, Waist Circumference, HDL-Cholesterol, Insulin Resistance and Mortality Risk in Adult Boston Community Dwellers of Puerto Rican Origin. *Am J Neuroprot Neuroregen.* 2011 Jun;3(1):48-52.

Oxenkrug GF, Requintina PJ, Mikolich DL, Ruthazer R, Viveiros K, Lee H, Summergrad P. Neopterin as a marker of response to antiviral therapy in hepatitis C virus patients. *Hepat Res Treat.* 2012;2012:619609.

Ozkan Y, Mete G, Sepici-Dincel A, Sepici V, Simsek B. Tryptophan degradation and neopterin levels in treated rheumatoid arthritis patients. *Clin Rheumatol.* 2012 Jan;31(1):29-34.

Park S, Kang S, Chen X, Kim EJ, Kim J, Kim N et al. Tumor suppression via paclitaxel- loaded drug carriers that target inflammation marker upregulated in tumor vasculature and macrophages. *Biomaterials* 2013; 34: 598–605.

Piccinini L, Zironi S, Federico M, Pini L, Luppi G. Urinary neopterin in malignant lymphoma. *Int. J. Biol. Markers* 6 (1991) 231–236.

Prasanna A, Ahmed MM, Mohiuddin M, Coleman CN. Exploiting sensitization windows of opportunity in hyper and hypo-fractionated radiation therapy. *J Thorac Dis.* 2014 Apr;6(4):287-302.

Putzki H, Aschern F, Henkel E, Heymann H. Neopterin. A tumor marker in colorectal carcinoma? *Dis Colon Rectum* 1987;30(11):879–83.

Pu Liu, Jade Jaffar and Karl Erik Hellstrom. Administration of cyclophosphamide changes the immune profile of tumor-bearing mice. *J Immunother.* 2010 Jan; 33(1): 53–59.

Quinn MA, Benedet JL, Odicino F, Maisonneuve P, Beller U, Creasman WT, Heintz AP, Ngan HY, Pecorelli S. Carcinoma of the cervix uteri. FIGO 26th Annual Report on the Results of Treatment in Gynecological Cancer. *Int J Gynaecol Obstet.* 2006 Nov;95 Suppl 1:S43-103.

Ramírez-Soria MP, España-Gregori E, Aviñó-Martínez J, Pastor-Pascual F. Blepharitis related to cetuximab treatment in an advanced colorectal cancer patient. *Arch Soc Esp Oftalmol.* 2008;83:665-68.

Ramos E, Suzuki S, Marks D, Inui A, Asakawa A, Meguid M. Cancer anorexia-cachexia syndrome: cytokines and neuropeptides. *Curr. Opin. Clin. Nutr. Metab. Care* 7 (2004) 427–434.

Reibnegger G, Bichler AH, Dapunt O, Fuchs DN, Fuith LC, Hausen A, et al. Neopterin as a prognostic indicator in patients with carcinoma of the uterine cervix. *Cancer Res* 1986;46:950–5.

Reibnegger G, Hetzel H, Fuchs D, Fuith LC, Hausen A, Werner ER, et al. Clinical significance of neopterin for prognosis and follow-up in ovarian cancer. *Cancer Res* 1987;47(18):4977–81.

Reibnegger G, Krainer M, Herold M, Ludwig H, Wachter H, Huber H. Predictive value of interleukin-6 and neopterin in patients with multiple myeloma. *Cancer Res.* 51 (1991) 6250–6253.

Reibnegger G, Fuchs D, Fuith LC, Hausen A, Werner ER, Werner-Felmayer G, et al. Neopterin as a marker for activated cell-mediated immunity: application in malignant disease. *Cancer Detect Prev* 1991;15:483–90.

Reissigl, H.; Rosmanith, P.; Schönitzer, D. (1989) Beitr. Infusionsther. 24, 14-17.
Abstract [cross reference]

Rose PG, Bundy BN, Watkins EB, Thigpen JT, Deppe G, Maiman MA, et al. Concurrent cisplatin-based radiotherapy and chemotherapy for locally advanced cervical cancer. *N Engl J Med* 1999;340:1144–53.

Sakurai A, Goto M. Neopterin: isolation from human urine. *J. Biochem.* 61 (1967) 142–145.

Sánchez-Regaña M, Catasús M, Creus L, Umbert P, Serum neopterin as an objective marker of psoriatic disease activity. *Acta Derm Venereol.* 2000 May;80(3):185-7

Schroecksnadel K, Winkler C, Fuchs D, Method for urinary neopterin measurements by HPLC., *J. Biochem. Biophys. Methods* 66 (2006) 99–100.

Schumacher M, Halwachs G, Tatzber F et al . Increased Neopterin in Patients With Chronic and Acute Coronary Syndromes. *Journal of the American College of Cardiology Volume 30, Issue 3, July 1997, Pages 703–707*

Shurin GV, Tourkova IL, Kaneno R, Shurin MR. Chemotherapeutic agents in noncytotoxic concentrations increase antigen presentation by dendritic cells via an IL-12-dependent mechanism. *J Immunol* 2009; 183: 137–144.

Signorelli SS, Anzaldi M, Fiore V, Candido S, Di Marco R, Mangano K, Quattrocchi C, Neri S. Neopterin: a potential marker in chronic peripheral arterial disease. *Mol Med Rep.* 2013 Jun;7(6):1855-8.

Spary LK, Al-Taei S, Salimu J, Cook AD, Ager A, Watson HA, Clayton A, Staffurth J, Mason MD, Tabi Z. Enhancement of T cell responses as a result of synergy between lower doses of radiation and T cell stimulation. *J Immunol.* 2014 Apr 1;192(7):3101-10

Sramek V, Melichar B, Studentova H, Kalabova H, Vrana D, Lukesova L, Adam T, Hlidkova E, Juranova J, Kujovska Krcmova L and Solichova D: Systemic immune response and peripheral blood cell count in patients with a history of breast cancer. *Pteridines 24: 211-217, 2013.*

Tanaka H, Matsushima H, Mizumoto N, Takashima A. Classification of chemotherapeutic agents based on their differential in vitro effects on dendritic cells. *Cancer Res 2009; 69: 6978–6986.*

Turgut T, Akbulut H, Deveci F, Kacar C, Muz MH. Serum interleukin-2 and neopterin levels as useful markers for treatment of active pulmonary tuberculosis. *Tohoku J Exp Med. 2006 Aug;209(4):321-8.*

Ursavaş A, Karadag M, Oral AY, Demirdogen E, Oral HB, Ege E. Association between serum neopterin, obesity and daytime sleepiness in patients with obstructive sleep apnea. *Respir Med. 2008 Aug;102(8):1193-7.*

Van Cutsem E, Kohne CH, Hitre E, Zaluski J, Chien CRC, Makhson A, D Haens G, Pinter T, Lim R, Bodoky G, Roh JK, Folprecht G, Ruff P, Stroh C, Tejpar S, Schlichting M, Nippgen J and Rougier P: Cetuximab and chemotherapy as initial treatment for metastatic colorectal cancer. *N Engl J Med 360: 1408-1417, 2009.*

Wachter H, Hausen A, Grassmayr K. Increased urinary excretion of neopterin in patients with malignant tumors and with virus diseases, Hoppe Seyler's *Z. Physiol. Chem. 360 (1979) 1957–1960*

Wachter H, Fuchs D, Hausen A, Reibnegger G and Werner ER: Neopterin as marker for activation of cellular immunity: immunologic basis and clinical application. *Adv Clin Chem 27: 81 - 141, 1989.*

Weiss G, Kronberger P, Conrad F, Bodner E, Wachter H and Reibnegger G: Neopterin and prognosis in patients with adenocarcinoma of the colon. *Cancer Res 53 : 260 - 265, 1993.*

Weiss G, Schroecksnadel K, Mattle V, Winkler C, Konwalinka G, Fuchs D. Possible role of cytokine-induced tryptophan degradation in anaemia of inflammation. *Eur. J. Haematol.* 72 (2004) 130–134.

Werner E, Bichler A, Daxenbichler G, Fuchs D, Fuith L, Hausen A, Hetzel H, Reibnegger G, Wachter H. Determination of neopterin in serum and urine. *Clin. Chem.* 33 (1987) 62–66.

Werner E, Werner-Felmayer G, Fuchs D, Hausen A, Reibnegger G, Yim J, Pfeleiderer W, Wachter H. Tetrahydrobiopterin biosynthetic activities in human macrophages fibroblasts, THP-1, and T 24 cells. GTP- cyclohydrolase I is stimulated by interferon-gamma, and 6- pyruvoyl tetrahydropterin synthase and sepiapterin reductase are constitutively present. *J. Biol. Chem.* 265 (1990) 3189–3192.

Widner B, Mayr C, Wirleitner B, Fuchs D. Oxidation of 7,8- dihydroneopterin by hypochlorous acid yields neopterin. *Biochem. Biophys. Res. Commun.* 275 (2000) 307–311.

Wiegele J, Margreiter R, Huber J, Dworzak E, Fuchs D, Haussen A, et al. Urinary neopterin excretion value in breast cancer patients. In: Pfeleider W, Wachter H, Blair JA, editors. *Biochemical and clinical aspects of pteridines*. Berlin: Walter de Gruyter; 1984. p. 417–24

Wirleitner B, Reider D, Ebner S, Boeck G, Widner B et al. Monocyte-derived dendritic cells release neopterin. *J. Leukocyte Biol.* 72 (2002) 1148–1153

Witek M, Blomain ES, Magee MS, Xiang B, Waldman SA, Snook AE. Tumor radiation therapy creates therapeutic vaccine responses to the colorectal cancer antigen GUCY2C. *Int J Radiat Oncol Biol Phys.* 2014 Apr 1;88(5):1188-95.

Xuanming Yang, Xunmin Zhang, Eric D Mortenson, Olga Radkevich-Brown, Yang Wang and Yang-Xin Fu. Cetuximab-mediated Tumor Regression Depends on Innate and Adaptive Immune Responses *Mol Ther.* 2013 January; 21(1): 91–100

Yang X, Zhang X, Mortenson E, Radkevich-Brown O et al. Cetuximab-mediated Tumor Regression Depends on Innate and Adaptive Immune Responses. *Mol Ther.* 2013 January; 21(1): 91–100

Yildirim Y, Gunel N, Coskun U, Pasaoglu H, Aslan S, Cetin A. Serum neopterin levels in patients with breast cancer. *Med Oncol.* 2008;25(4):403-7.

Zangerle R, Reibnegger G, Wachter H, Fuchs D. Weight loss in HIV-1 infection is associated with immune activation, *AIDS* 7 (1993) 175–181

Zangerle R, Schönitzer D, Fuchs D, Möst J, Dierich M.P. and Wachter H. Reducing HIV transmission by seronegative blood , *Lancet*, (1992) 339, 130-131 Abstract

Zhang W, Gordon M, Schultheis AM, Yang DY, Nagashima F, Azuma M, Chang HM, Borucka E, Lurje G, Sherrod AE, Iqbal S, Groshen S and Lenz HJ: ECGR2A and FCGR3A polymorphisms associated with clinical outcome of epidermal growth factor receptor-expressing metastatic colorectal cancer patients treated with single-agent cetuximab. *J Clin Oncol* 25: 3712-3718, 2007.

12. Appendices

FIGURES

FIGURE 1. Neopterin: 2-amino-4-oxo-6-(derythro-1', 2', 3'-trihydroxypropyl)-pteridine

FIGURE 2. In various cells the Th1-type cytokine IFN-gamma (IFN- γ) induces the GTP-cyclohydrolase I (GCH I) to produce 7, 8-dihydroneopterintriphosphate. Due to a deficiency in 6-pyruvoyltetrahydropterin synthase (PTPS) in human monocyte-derived macrophages and dendritic cells, the production of 5, 6, 7, 8-tetrahydrobiopterin is almost zero and neopterin is produced in high concentrations. Ref Becker et al.

FIGURE 3: IFN-Gamma induce production of ROS, Neopterin and TNF alpha

FIGURE 4: Induction of neopterin formation in brain cells. Proinflammatory cytokines like interferon- γ (IFN- γ) induce expression of GTP-cyclohydrolase I in various brain cells. As an intermediate product 7, 8-dihydroneopterin-triphosphate is produced which is further converted by pyruvoyl-tetrahydropterin synthase (PTPS) to form 5, 6, 7, 8-tetrahydrobiopterin (BH₄), the cofactor of several aromatic amino acid monooxygenases that are involved in the production of tyrosine, L-DOPA, serotonin and nitric oxide. Different from neurons, monocytic cells possess only low constitutive activity of PTPS. Thus, 7, 8-dihydroneopterin-triphosphate does not undergo conversion to BH₄, rather it is dephosphorylated and oxidized to neopterin in non-enzymatic reactions.

FIGURE 5. HPLCC apparatus and setup

FIGURE 6: The proposed immune modulatory mechanism of targeted agent (cetuximab)

FIGURE 7. Number of samples collected in Part 1 of the study with patients in Group A. Figure 7 shows the sample number collected serially during the treatment in study group A

FIGURE 8. Patients of cohort A in whom serial urinary samples were collected were divided in two groups based on High and Low Neopterin/Creatinine ratio (214 as the cut off) are shown in Figure 8

FIGURE 9. The changes in urinary neopterin concentrations during the course of therapy in patients 1-6 of cohort A are shown in Figure 9.

FIGURE 10. The changes in urinary neopterin concentrations during the course of therapy in patients 7-12 of cohort A are shown in Figure 10.

FIGURE 11. The changes in urinary neopterin concentrations during the course of therapy in patients 13-18 of cohort A are shown in Figure 11.

FIGURE 12. The changes in urinary neopterin concentrations during the course of therapy in patients 19-24 of cohort A are shown in Figure 12.

FIGURE 13. The changes in urinary neopterin concentrations during the course of therapy in patients 25-30 of cohort A are shown in Figure 13.

FIGURE 14. The changes in urinary neopterin concentrations during the course of therapy in patients 31-36 of cohort A are shown in Figure 14.

FIGURE 15. The course of urinary neopterin concentrations during the course of therapy in a 78-year-old patient (patient no 9 of cohort A) treated with single-agent cetuximab is shown above/ in Figure 15.

FIGURE 16. Stacked line graph showing trends in patients with Neopterin/Creatinine < 214 in Group A. The stacked lines showing trend of changes in urinary neopterin levels in patients with pretreatment neopterin <214 are shown in Figure 16. A general rise in neopterin levels was noted on commencement of anticancer therapy.

FIGURE 17. Stacked line graph showing trends in patients with Neopterin/
Creatinine > 214 in Group A. The stacked lines showing trend of changes in urinary
neopterin levels in patients with pretreatment neopterin >214 are shown in Figure 17.
A general fall in neopterin levels was noted on commencement of anticancer therapy.

FIGURE 18. Correlation between urinary neopterin and hemoglobin in patients of
group A

FIGURE 19. Correlation between peripheral blood leukocyte count and urinary
neopterin concentrations in patients of group A

FIGURE 20. Correlation between carcinoembryonic antigen and urinary neopterin
concentrations in cohort A

FIGURE 21. Survival curves cohort A - At the time of the analysis, 44 patients died while
one patient was alive after 74 months. Survival of patients with urinary neopterin
concentration of 214 [$\mu\text{mol/mol}$ creatinine] or above was significantly inferior compared
to patients with initial urinary neopterin below 214 [$\mu\text{mol/mol}$ creatinine] (median 10.1
vs. 17.7 months, $p < 0.05$; Figure 21).

FIGURE 22 – Patients characteristics in cohort B. The division of patients in cohort B
based on diagnosis is demonstrated in Figure 22

FIGURE 23- Number of samples analyzed the part 2 of the study on patients in
cohort B. Number of daily samples analyzed the part 2 of the study on patients in
cohort B is shown in figure 23

FIGURE 24 The course of urinary neopterin concentrations in patients 1 and 2 cohort B is shown.

FIGURE 25. Urinary neopterin concentrations during the course of chemoradiation in patients 3 to 6 of cohort B

FIGURE 26. Urinary neopterin concentrations during the course of chemoradiation in patients 7 to 10 of cohort B

Tables

Table 1: Neopterin concentrations (mean + S.D. and 97.5th percentiles) in urine of healthy individuals

Table 2: depictions of evidence in literature on tumor types and the use of neopterin in prognosis (Table modified from Murr et al, 2002. based on new evidence)

Table 3 .The mean \pm standard deviation of neopterin levels in the two groups ^a $p < 0.05$, ^b $p < 0.01$, ^c $p < 0.001$, ^d $p < 0.0001$. The standard deviations of neopterin in high and low neopterin level groups are shown in table 3

Table 4a: Serial Urinary neopterin levels during the systemic therapy in group A

Table 4b : Pretreatment neopterin in patients of cohort A in whom serial sample collections could not completed

Table 5. The cohort of patients studied in cohort B. Age group, histology and staging

Table 6. Urinary neopterin in patients of cohort B during the course of pelvic radiation . Shown are the mean \pm standard deviation (SD) of interval between the treatment start and sample collection, number of patients examined, mean \pm SD of urinary neopterin and p -value (Wilcoxon signed rank test) at the respective visit.