Charles University in Prague
Faculty of Medicine in Hradec Králové

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 aktivace, v odhadu prognózy, monitorování odpovědi na léčbu a komplikací u pacientů s nádorovým onemocněním

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Chairperson of the Commission for Dissertation Defences
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1. 1 Souhrn

Název práce
Klinické použití neopterinu, laboratorního biomarkeru imunitní aktivace, v určení prognózy, monitorování odpovědi na léčbu a komplikací u pacientů s nádorovým onemocněním
Uvod
Neopterin je biomarker imunitní aktivace, 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, podstupujích 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 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.

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. It is now evident that cancerous cells manage to escape immune recognition and elimination [Dranoff 2004]. This quality of cancerous cell is known to be an important aspect in pathogenesis of malignant disease [Boon and Der Bruggen 1996]. In addition, the crosstalk between normal and neoplastic cells 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 component and the products of host response in tumor microenvironment and also in various visceral fluids [Melichar et al 1998].

IFN-gamma is a cytokine produced by the T-lymphocytes and natural killer cells. It has also been found to play an important role in regulating tumor growth [Ikeda et al 2002]. For example IFN-y plays roles in expressing the antitumor efficacy of IL-1 by supporting the T-cell acceptability of tumor masses and mediating the antitumor effects of migrated T cells. Thus serum concentrations of IFN-gamma could reflect the systemic immune response [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 [Mountabarrik et al 1994] also produce neopterin. However, neopterin output from monocyte/macrophages was found to be higher several folds to several orders of magnitude [Melichar et al 2006a]. By measuring neopterin not only the extent of cellular immune activation, but also the extent of oxidative stress can be estimated [Murr et al 2006, 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]. In the early 1980's Fuchs et al. proposed neopterin to be a marker of immune activation [Fuchs et al 1984]

Chemistry and synthesis

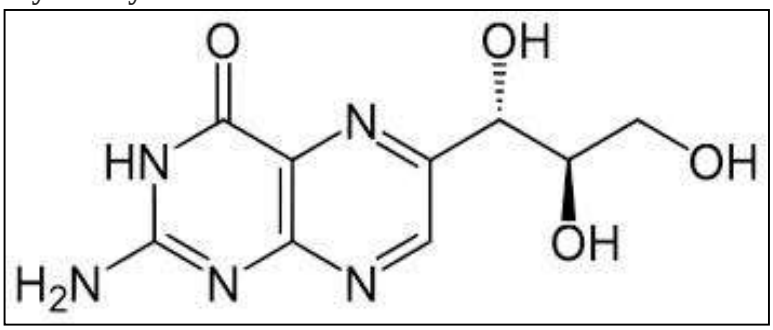

FIGURE 1. Neopterin: 2-amino -4-oxo-6 –(derythro-1', 2', 3'-trihydroxypropyl)-pteridine [1]

Chemically, neopterin is 2-amino -4-oxo-6 –(derythro-1', 2', 3'-trihydroxypropyl)-pteridine, which is an unconjugated pteridine. It is synthesized from

GTP (Guanosine Triphosphate) 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 the location. Pyruvoyltetrahydropterin synthase (PTPS) is the enzyme responsible for conversion of 7, 8- dihydroneopterintriphosphate to 5,6,7,8 – tetrahydrobiopterin (BH4).

IFN gamma can stimulate the production of GCH-1 in various cell types. In cells other than macrophages GCH-1 activity remains lower than the PTPS synthase activity leading to synthesis of BH4 [Werner et al 1990]. Thus monocytes/macrophages remain the most important cell population responsible for neopterin production [Leitner et al 2003]. Neopterin is produced out of the 7, 8-dihydroneopterintriphosphate intermediate at the expense of 5, 6, 7, 8-tetrahydrobiopterin (BH4)[Fuchs et al 1994a].

7, 8- dihydroneopterintriphosphate is converted to neopterin by the dephosphorylation and further oxidation [Widner et al 2000, Gieseg et al 2001]. 7, 8-dihyrdoneopterin 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].

Elimination –

Neopterin is eliminated by the kidney [Fuchs et al 1994b] and a strong correlation between urinary and serum neopterin level has been established [Fuchs et al 1988a]. 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]. 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 small molecular mass (253 and 255 D) [Murr et al 2002]. Neopterin is sensitive to direct sunlight and irradiation and therefore samples must be protected from both [Laich et al 2002].

Table 1 Neopterin in urine (μmol/mol creatinine): Normal level

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 [www.neopterin.net].

In serum and plasma 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]. HPLC measurements of neopterin concentrations in serum or plasma are limited by the fact

that protein precipitation will influence neopterin levels [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].

3. Background

Neopterin in human pathology

Inflammation has been linked to the development of cancer [Hagemann et al 2007, Laird et al 2011] and is now accepted as one of the hallmarks of cancer [Colotta et al 2009]. An increased risk of malignancy is associated with the chronic inflammation [Balkwill and Mantovani 2001]. Chronic inflammation invariably leads to a chronic immune stimulation.

Hence, the study biomarkers to monitor the immune activity can provide an insight in cancer development and its management. However, the clinical utilization of biomarkers associated with host response to malignancy has been limited [Melichar 2013].

Neopterin and chronic immune stimulation

As the production of IFN- gamma is enhanced by pro-inflammatory cytokines, systemic concentrations of neopterin may be influenced both systemic immune and inflammatory response [Wachter et al 1989].

The general state of immune activation in patients with advanced cancer may be a consequence of the failure of the host immune system to cope with the tumor [Melichar et al 2006a]. We also 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 [Baier-Bitterlich et al 1995]. Thus chronic immune activation may lead to a qualitative and quantitative impairment of the host defense

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

The sensitivity of the urine neopterin in diagnosis varies according to the type and stage of cancer [Byram et al 2004]. This sensitivity may reache 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 frequency of increased neopterin varies is in other cancers such as gynecologic cancers [Reibnegger et al 1986, Reibnegger et al 1987], lung cancers [Conrad et al 1987], cancers of colon ranges over 50% [Putzki et al 1987] and in breast cancer it has been found to be much lower at around 20% [Wiegele et al 1984].

Neopterin concentrations correlate with tumor stage of lymphomas and might be considered as a prognostic marker [Hausen et al 1981, Piccinini et al 1991] as well as in patients with multiple myeloma [Reibnegger et al 1991].

An increased urinary neopterin excretion was found to be a sign for 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]. There are suggestions that neopterin may an indicator of metastatic disease [Yildrim et al 2008].

Anticancer therapy, immune activation and neopterin

Several studies have described effects of cytotoxic drugs on macrophages, dendritic cells (DCs) and natural killer (NK) cells. In animal experiments oral administration of metronomic (low dose) cyclophosphamide lead to a restoration of

peripheral T cell proliferation and innate killing activities [Ghiringhelli et al 2007]. Cyclophosphamide [Pu et al 2010] and 5 FU [Khallouf et al 2012] were found to have a positive impact on the cellular components of immune response.

Cyclophosphamide metabolites were found to increase the tissue associated macrophages (TAM-M1) and thus could significantly increase the specific immune response as well as nonspecific innate reaction [Brynarski et al 2009]. Similarly, paclitaxel can stimulate TAMs cytotoxicity directly [Park et al 2013] and induce the activation of dendritic cells (DCs), NK and tumor-specific CTL via the secretion of IL-12 and TNF-a and inducible nitric oxide synthase (iNOS) [Javeed et al 2009] resulting in tumor regression [Bracci et al 2014].

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]. Low noncytotoxic concentrations chemotherapeutic agents do not induce apoptosis of DCs but directly enhance DC maturation and function [Kaneno et al 2009]. Paclitaxel, doxorubicin and methotrexate can promote the ability of murine BM–DCs to present antigens to T-cells in vitro [Shurin et al 2009]. 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].

Radiation has also been shown to affect the neopterin levels [Holečková et al 2013]. A study was able to demonstrate that α irradiation could be an immunogenic cell death inducer [Gorin et al 2014]. 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]. Optimal sequencing of RT and immunotherapy may amplify antigen-specific local and systemic immune responses [Witek et al 2014]. RT may 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]. These changes may influence systemic neopterin levels.

Though we have significant data on neopterin and conventional anticancer treatment, the information of neopterin and targeted biological therapy is limited. The precise mechanisms by which the targeted agents such as cetuximab work also appear to be more complex than previously thought.

In initial studies activity of cetuximab in combination with chemotherapy in the first line of treatment of metastatic colorectal carcinoma was demonstrated in some patients with tumors not harboring RAS mutation [Bokemeyer et al 2009, Van Cutsem et al 2009]. Thus underlining an alternative mechanism of action of this agent. 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]. It was found that *in vivo* antitumor activity of cetuximab could be associated with a complement-mediated immune response [Hsu et al 2010]. It has been 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[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 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].

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.

Neopterin and weight-loss and cachexia

The presence of cachexia is a significant risk factor and an indicator of negative prognosis in cancer patients [Ramos et al 2004]. Cachexia is a catabolic state mediated by multiple factors such as TNF-alpha {cachectin}, IFN- gamma and even neopterin that may work individually or in conjunction [Moldawer et al 1997]. TNF-alpha IFN-gamma and leukemia inhibitory factor act as cachectins in animal models [Kurzrock et al 2001].

In a study with patients with HIV infection a correlation between body mass index, urinary neopterin, development of AIDS-defining infections, weight loss, and a decline in CD4+ T-cell count was observed [Zangerle et al 1993]. Extrapolating from these 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.

Neopterin and Anemia

Neopterin levels were found to be elevated in cancer related anemia [Fuith et al 1989, Weiss et al 2004] with an inverse correlation of the urinary neopterin levels with hemoglobin levels [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 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]. Increase in urinary neopterin levels significantly preceded increases in fatigue intensity with a temporal delay of 60—72 hours [Haberkorn et al 2013].

Potential use of neopterin in reflecting complications

Neopterin can also reflect the systemic immune and inflammatory responses in various other human disorders. Several studies have demonstrated use of neopterin in infections [Cesur et al 2005], 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]. Findings suggest that the pretreatment level of neopterin might be used in routine clinical practice to predict the response to antiviral therapy in HCV patients [Oxenkurg et al 2012]. Determination neopterin levels in chronic inflammatory disease may provide a better understanding of progression of these diseases [Ozkan et al 2012].

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 was observed between baseline parameters of intestinal permeability and urinary neopterin [Dvorak et al 2010].

Neopterin is significantly increased in patients with chronic coronary artery disease [Schumacher et al 1997] and may represent early marker for post-traumatic complications [Mommsen et al 2009]. Screening blood for neopterin concentrations improved safety of blood transfusions [Murr et al 2002]. Higher neopterin levels also correlated with acute rejection in the first year post-transplant [Carey et al 2013].

Neopterin in therapy related complications

Urinary neopterin concentrations were found to be relatively stable in cancer patients in the absence of complications [Melichar et al 2007a]. Data has demonstrated an association between increased urinary neopterin concentrations and age or presence of comorbid diseases in patients with breast carcinoma. [Melicharova et al 2010]. In a noteworthy study on patients of head and neck tumor neopterin was significantly increased with therapy related toxicity such as nausea, mucositis and performance status [Holečková et al 2013]. 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 could possibly be used in clinical settings to monitor immune activity in cancer patients.

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 address the association 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 performed at the University Hospital in 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.

The investigations were carried out at the University Hospital in Hradec Kralove. The patients were enrolled at the Department of Oncology and Radiotherapy FNHK. The determination of urinary neopterin was performed in the laboratory of the Third Department of Medicine – Gerontology and Metabolic Care.

6. Study

6.1 Part I

6.1.1 Patient group- Cohort A

Diagnosis of patients in cohort A -Metastatic colorectal carcinoma

Patient characteristics

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

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/m^2, subsequently 250 mg/m^2 weekly) followed by irinotecan (180 mg/m^2), leucovorin (200 mg/m^2), and 5-fluorouracil (400 mg/m^2 bolus and 1200 mg/m^2 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.

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. The samples were transferred into a refrigerator at temperature of -20^o C. These were analyzed at the laboratory based at the University Hospital in Hradec Kralove.

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 5μ, C18, 150 × 3 mm (Phenomenex, Torrance, CA, USA) at 25°C, injection volume was 1μL. 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 (μmol/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 (μmol/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.

6.1.3 Statistics

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 urinary neopterin concentration of 214 [µmol/mol creatinine] or higher
2. Those with initial urinary neopterin below 214 [µmol/mol creatinine]

Summary of results of part I

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

Daily neopterin measurements were obtained from 36 patients. 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 µmol/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 µmol/mol Creatinine. In the patient treated with single-agent cetuximab, an increase of urinary neopterin was observed despite elevated initial neopterin concentrations.

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 µmol/mol creatinine or above was significantly inferior compared to patients with initial urinary neopterin below 214 µmol/mol creatinine (median 10.1 vs. 17.7 months, p<0.05)

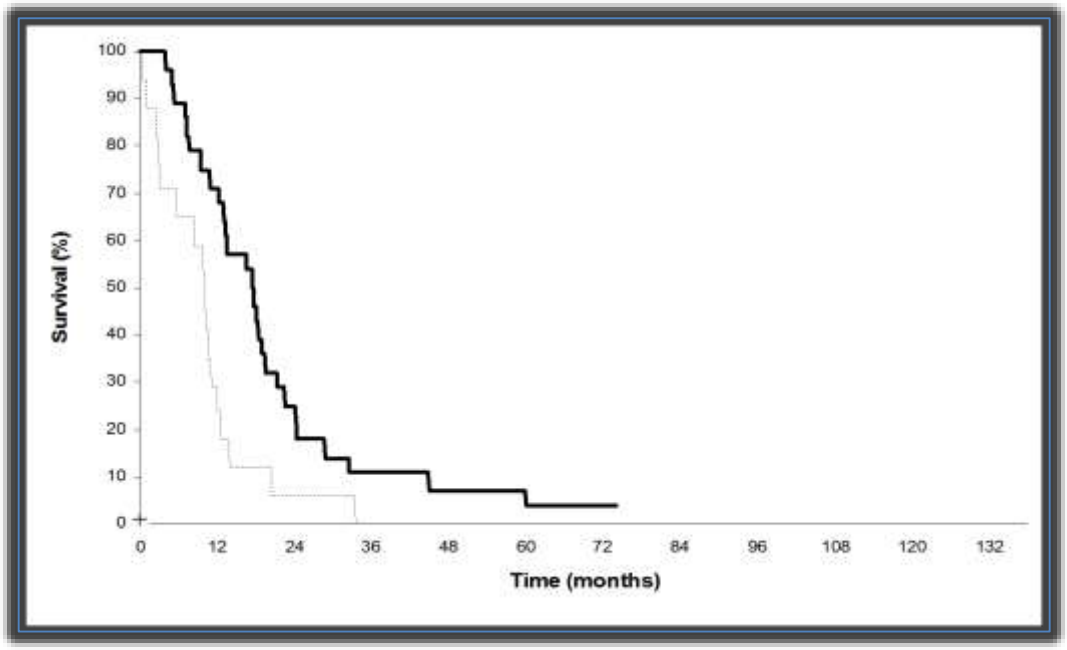

FIGURE 2. Survival curves cohort A

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 [µmol/mol creatinine] or above was significantly inferior compared to patients with initial urinary neopterin below 214 [µmol/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

Number of patients (n) = 10

Diagnosis -

1. Patients with carcinoma of uterine cervix n= 9
   FIGO Stage IIIB n= 6 , Stage IIB n = 3
2. Patients with carcinoma of the vulva n = 1

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 1).

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/m^2) 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 delivered to 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.

6.2.2Methodology

Methodology has been described in Part 1 of the study

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

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 µmol/mol creatinine 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).

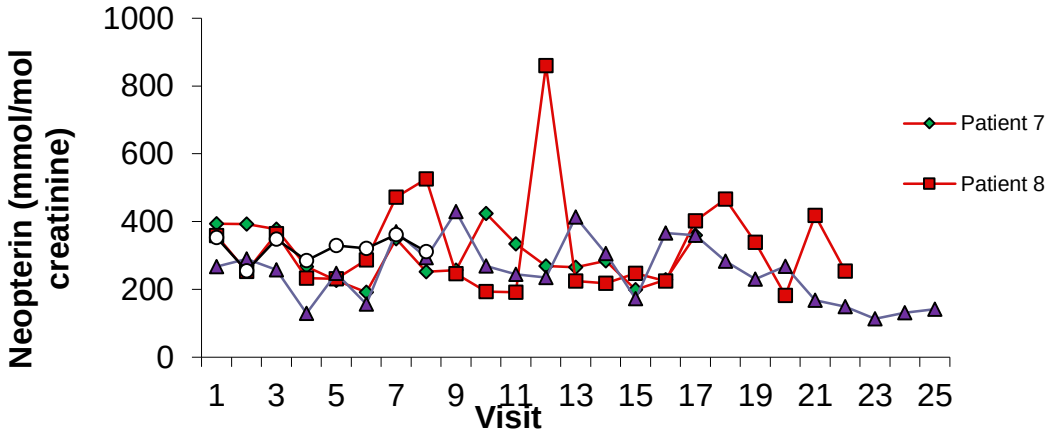

Figure 3 – Neopterin spike in patient 8 on day 12 coincided with a skin rash. (example)

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 systemic neopterin concentrations has been demonstrated across a spectrum of malignant disorders [Reibneggar et al 1991]. 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 also with radiotherapy. So far the information about neopterin in patients treated with targeted agents such as cetuximab is limited.

Our data was able to demonstrate:

1. That the pretreatment neopterin level 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.

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.

We were also able to identify a correlation between urinary neopterin, peripheral blood leukocyte count, hemoglobin, and CEA concentration. Our study has corroborated the prior reports on correlation of neopterin concentrations with various laboratory parameters. The inverse correlation of hemoglobin with neopterin concentrations in previous studies [Melichar et al 2008a, Fuchs et al 1991, Sramek et al 2013] has also been demonstrated in our cohort A. In a study on 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.

The precise the mechanism of action of targeted agent such as cetuximab is not entirely clear. In addition to targeting the intracellular pathways it may involve, at least partly, the activation of the immune response.

Cetuximab could trigger antibody-dependent cell-mediated cytotoxicity [Messersmith et al 2007]. 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.

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].

Rise in daily neopterin measurements organ transplant recipients was an early indicator of acute complications [Chin et al 2008]. There have been reports showing that increase in neopterin concentrations preceded complications while a decrease in urinary neopterin was associated with tumor control [Melichar et al 2007]. These complications can also be 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. Neopterin may offer a tool to monitor such important but subtle complication.

The best example of this potential is the successful implementation of neopterin for clinical practice has been in the area of transfusions. Neopterin was found to be an

excellent marker to screen acute infective phases in the donor blood. [Operkalski et al 1997]. Screening of blood donations with neopterin assisted in the detection and exclusion of viral infections [Zangerle et al 1992, Reissigl et al 1989]. In this way subclinical infections or silent systemic disorders may be detected in a higher frequency and increase the safety of transfusion [Hönlinger et al 1989].

In case of management of cancer patients the complications related or unrelated to therapy could, have a huge impact on the overall outcome of treatment by causing unwanted delays, dose reduction, hospital admissions and increased mortality. This can derail the treatment plan either in part or in its entirety. With further data to 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.

In our study, we were unable to detect any significant change 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 of similar size [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 of 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 neopterin. Hence, we can say that in cohort B the rise of neopterin reflected the emergence of the complications of 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 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]. 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.

Neopterin levels do reflect immune activation in cancer patients however it lacks the specificity required to formulate a strategy in cancer subtypes. However, several problems associated with different malignancy and with different anticancer treatment are common and their manifestations may be 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. We have described these limitations in section 9. Consequently, statistical analyses performed here have to be regarded as exploratory at best.

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

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

We found that in cohort A, a higher neopterin levels were associated with poor prognosis. Patients whose neopterin levels were higher 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. We were also 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.

We monitored neopterin in patients of different kinds of malignancies while undergoing all established non-surgical anticancer therapy including chemotherapy, radiotherapy and targeted therapy.

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.

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.

. It must be added that much work with stronger evidence will be needed to sieve out clinical applications of neopterin in the clinical setting. Our study may provide a building block in this exercise.

9. Limitations of the current study

Our study has successfully demonstrated that neopterin can potentially play a role in clinical settings however, it 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.

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. Further research may shed light on why certain patients undergoing chemoradiotherapy do not show changes in neopterin levels.

10. Recommendations

Our understanding of immune system and its role in cancer development 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.

Our research has also thrown up many questions that need further investigation. 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 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.

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12. Publications and Lectures

Original articles

Prognostic factors and treatment outcomes in patients with Small Bowel Adenocarcinoma (SBA): the Royal Marsden Hospital (RMH) experience. Khan K, Peckitt C, Sclafani F, Watkins D, Rao S, Starling N, Jain V, **Trivedi S**, Stanway S, Cunningham D, Chau I. *BMC Cancer. 2015 Jan 21;15:15.* ***(IF 3.36)***

Urinary neopterin concentrations during combination therapy with cetuximab in previously treated patients with metastatic colorectal carcinoma. Bohuslav Melichar Hana Kalábová, Lenka Kujovská Krčmová, **Sachin Vipin Trivedi**, Pavlína Králíčková, Eva Malířová, Miroslav Pecka, Hana Študentová, Michaela Zezulová, Petra Holečková, Dagmar Solichová. In Vivo September-October 2014 vol. 28 no. 5 953-959 **(*IF 1.148)***

Urinary neopterin concentrations during radiotherapy for gynecological cancer. **Sachin Vipin Trivedi**, Bohuslav Melichar, Martin Majirský, Pavel Veselý, Hana Kalábová, Hana Študentová, Lenka Kujovská Krčmová, Dagmar Solichová and Martin Doležel. *Pteridine, Volume 25, Issue 1 (Apr 2014)* ***(IF 0.326)***

Physician perspectives on resuscitation status and DNR order in elderly cancer patients, **Sachin Trivedi**. *Reports of Practical Oncology & Radiotherapy Vol. 18-1,53-56, Jan. 2013*

(IF 0.717)

The search for the practical methods of intervention in reducing the psychosocial impact while dealing with cancer as a disease - A clinician's point of view **S Trivedi**, J Petera, S Fillip, Z Hrstka , *J Cancer Res Ther. Oct-Dec;3(4):193-7. 2007* ***(IF 0.791)***

Overview articles and case reports

Metastatic carcinoma of breast or a chordoma? A case report and clinical perspectives. Trivedi S, Odrazka K. J Can Res Ther 2015;11:645 **(IF 0.791)**

Carcinoid tumors metastasis to the medial rectus muscle: A case report
Sachin Trivedi, Alice Dewdney, Michael Brada and Sheela Rao . (Submitted)

Lectures and poster presentations

Management of acute oncological problems – Core medical teaching at Queen Elizabeth Hospital Birmingham , December 2015

Carcinoma of Unknown Primary – Grand Round at New Cross Hospital , Wolverhampton , July 2015

Lecture on head injury in adult setting. *NICE guideline Audit, University hospital Birmingham UK 2011*

Lecture on tuberculosis guidelines – *Department of pediatrics, Royal Alexandra pediatric hospital, 2010*

Neopterin in Oncology – *Oncology academic meeting at Royal Wolverhampton NHS Trust July 2014*

Fifteen year experience of all the patients (pts) with small bowel adenocarcinoma (SBA), treated in a specialised gastrointestinal (GI) Oncology unit: a Royal Marsden Hospital (RMH) experience ; GI ASCO September 2013

Promoting the clinician to adopt measures of psychosocial intervention in cancer care. Study about choice of patient admission setting for cancer patients, an attempt. Fifth International conference of Society of Integrative Oncology; Atlanta Nov 20-21 2008,