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Differential Scanning Calorimetry, as a New Method to Monitor Human Plasma in Melanoma Patients with Regional Lymph Node or Distal Metastases

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1. Introduction

Cutaneus malignant melanoma (MM) is a highly malignant tumour of the skin and is responsible for more deaths than any other skin cancer (Imko-Walczuk et al., 2009). Melanocytes originate from the neural crest and in contrast to Langerhans' cells are located amongst the basal layer of the epidermis, hair bulb, eyes, ears, and meninges (Bandarchi et al., 2010; Fitzpatrick, 1971; Nordlund & Boissy, 2001). The pigmentary system of the skin is a complex set of reactions with many potential sites for dysfunction (Grichnik, 1998). Melanin pigment is produced by melanocytes in their specific cytoplasmic organelles called melanosomes. MM arises from the malignant transformation of melanocytes at the dermal-epidermal junction or from the nevomelanocytes of melanocytic nevi that become invasive and may metastasise.

The incidence of MM has been increasing in white populations. Although MM comprises less than 5% of malignant skin tumours; however, it is responsible for almost 60% of lethal skin neoplasia. With increased life expectancy of the elderly population, melanoma will be a public health challenge (Riker et al., 2010). Increased incidence of melanoma is partly due to early detection (thin melanomas) and partly due to true increase of incidence. Despite the increase in the incidence of melanoma, the prognosis has been improving due to earlier diagnosis of thin melanomas and hence in a curable stage (MacKie, 2000). The incidence of melanoma is equal in men and women and uncommon in children although there are reports that the incidence may be higher in women. A typical patient is usually a Caucasian adult in the 4th decade of life with lesion on the back and leg in male and female, respectively. One typical study revealed that the most common sites in decreasing order are the trunk (43.5%), extremities (33.9%), acral sites (11.9%), and head and neck (10.7%) (Bandarchi et al., 2010).

There is a complex interaction of environmental (exogenous) and endogenous factors. Up to 65% of MM is sun-related (Whiteman & Green, 1999). It is now widely accepted that the major environmental risk factor for the development of primary cutaneous melanoma is Ultraviolent (UV) radiation, which can be subdivided into UVA, UVB, and UVC. UV radiation in sunlight is cytotoxic and, in over dosages, clearly detrimental cells die in

apoptosis („sunburn cells") and strong inflammation occurs (Imko-Walczuk et al., 2009). The list of risk factors in developing MM includes pale skin, blond or red hair, numerous freckles and tendency to burn and tan poorly, presence of more than 50 acquired nevi, more than five dysplastic (atypical, Clark's) nevi, large congenital nevi, nevi larger than 6 mm, PUVA therapy, tendency to sunburn and tan poorly, use of tanning salons, Xeroderma pigmentosum, immunosuppression, chemical exposures, scars, Marjolin's ulcer, and genetic factors (Bandarchi et al., 2010; Halpern et al., 1991).

2. Diagnosis of melanoma malignum

2.1 Routine diagnostic methods and prognostic factors

During the past 30 years, there has been significant evolution in the diagnosis of early melanoma. Several factors have contributed to a marked improvement in detection of cutaneous melanomas at an early, curable stage (Rigel & Carucci, 2000). Early detection of MM remains the key factor in lowering mortality from this cancer. Recognizing the importance of this issue 25 years ago, Rigel's group at New York University published the mnemonic "ABCD" to facilitate the early diagnosis of melanoma (Rigel et al., 2010). The ABCD rule of typical MM means: asymmetry, border irregularity, colour variegation, diameter more than 6 mm. However, many exceptions may occur as they may do in other medical disciplines. Studies have demonstrated the usefulness of this paradigm in enhancing early melanoma diagnosis as a part of clinical examinations, mass screenings, and public education programs. Even though dermascopy, even in the hands of a relatively inexpert practitioner, may show high diagnostic accuracy and boost the clinical suspicion in diagnosing MM; however, the definitive diagnosis is confirmed done by biopsy.

The clinicopathological stage of the melanoma patients can determine by pathological evaluation of the primary lesion and of the dissected lymph nodes, as well as by routine examinations (lactate dehydrogenase test, chest X-ray, ultrasound of the abdominal cavity, and computed tomography (CT) or positron emission tomography combined with CT) (Neila & Soyer, 2011).

There are three classes of adverse prognostic factors in melanoma: pathological, clinical, and other factors including genetic alteration (Bataille, 2000). Among others the first group includes increasing the Breslow thickness and Clark level. In 1969, Clark et al. proposed staging criteria for lesions on the basis of skin invasion levels (Clark et al., 1969). Subsequently, Breslow evidenced the importance of the primary melanoma thickness in millimetres, and this index became one of the most important prognostic indicators, in association with data on ulceration, mitosis, regression, microscopic satellites, histopathologic subtype, and presence of vertical growth phase (Breslow & Macht, 1977; Byers & Bhawan 1998; Clemente et al., 1996). Proliferation of the primary melanoma as defined by the mitotic rate was identified as a powerful and independent predictor of survival. As a result, primary tumour mitotic rate is now a required element for the seventh edition melanoma staging system. Multiple thresholds of mitotic rate were examined statistically, and the most significant correlation with survival was identified at a threshold of at least 1/mm^2. Data from the American Joint Committee on Cancer Melanoma Staging Database demonstrated a highly significant correlation between increasing mitotic rate and declining survival rates. In a multifactorial analysis of 10,233 patients with clinically localized melanoma, mitotic rate was the second most powerful predictor of survival, after tumour thickness (Balch, et al., 2009). Clinical adverse factors include increasing age, male,

location of the lesion, and metastasis. Moreover, regional lymph node (Sentinel) status has emerged as an accurate method for evaluating the draining lymph node basin, allowing for the generation of valuable prognostic information (Mraz-Gernhard et al., 1998). Sentinel lymph node biopsy became a compulsory phase for patients with tumour thickness > 1 mm (Patnana, et al., 2011; Petrescu, et al., 2010). Beside routine clinical follow-up with the unaided eye additional techniques are being used to follow these high risk patients sequentially. Recently, there is a need for more studies to diagnose and monitor MM patients in any different clinical stages (as far as possible) with non-invasive methods.

2.2 New diagnostic method: A DSC technique

Differential Scanning Calorimetry (DSC) is a thermoanalytical technique which monitors small heat changes between a sample and reference. The technique was developed by Watson and O'Neill in 1960 (Watson & O'Neill, 1966). For biological samples, a dilute aqueous solution of a biomolecule is loaded into a sample chamber and a matched reference buffer loaded into a reference chamber. Both chambers are heated, and as the temperature increases, thermally-induced processes occurring in the sample cell result in heat either being absorbed or released. This creates a thermal imbalance between the sample and reference chambers which is compensated for by electrically-powered feedback heaters. This electrical power output is directly proportional to the apparent heat capacity of the sample and reference solutions and is the raw data recorded during a DSC experiment (Biltonen & Freire, 1978; Brandts & Lin, 1990).

The DSC thermogram, is an unique signature for bio-molecules reflecting the normal or pathomorphological changes under given solution conditions. Therefore, DSC technique allows demonstrating the thermal consequences of conformation changes in different bio-molecules (Zielenkiewicz, 2011). Moreover, DSC is useful method to evaluate local and global conformation changes in the structure of different tissue elements not only in the animal experiences, but in the orthopaedic and traumatologic, surgical, oncological and dermatological clinical studies (Ferencz, et al., 2011; Szántó & Lőrinczy, 2011; Wiegand, et al., 2011).

Depending on the structural nature of the protein, denaturation might reflect the independent melting of individual domains within the tertiary structure of the protein resulting in a complex thermogram with multiple transitions. A primary DSC thermogram is an extensive property of a protein solution and is therefore directly proportional to the mass of protein in solution. For plasma, assuming there are no significant interactions between the plasma proteins, the DSC thermogram will reflect the melting of a complex mixture of proteins with the observed thermogram representing the sum of the individual protein thermograms weighted according to their mass in solution (Garbett, et al., 2009). The high sensitivity of DSC towards binding interactions means that dramatic shifts in DSC thermograms can result from binding interactions. This represents an intrinsic advantage of the DSC method over other methods applied to the study of the plasma protein, such as electrophoresis and mass spectrometry.

The result of a DSC experiment is a curve of heat flux versus temperature or versus time. As the temperature increases the sample eventually reaches its melting temperature (T_m). The melting process results in an endothermic peak in the DSC curve. A biomolecule in solution is in equilibrium between the native (folded) conformation and its denatured (unfolded) state. The higher the thermal transition midpoint, when 50% of the biomolecules are unfolded, the more stable the molecule. The ability to determine T_m temperatures (T_1, T_2) and enthalpies

makes DSC a valuable tool in producing phase diagrams for various chemical systems. $T_{1/2}$ indicates the cooperatively of thermal domens. The calorimetric enthalpy (ΔH) is an absolute measurement of the heat energy uptake, given by the area under the transition peak. It depends on the total amount of (active) protein in the calorimeter cell (Fig. 1.).

Recently, numerous articles confirmed that DSC is widely used as a new diagnostic method for detection of diseases' seriousness, and as an applicable technique during monitoring of patients. Garbett and co-workers demonstrated in these calorimetric studies average thermograms for individuals diagnosed with various diseases (Lyme disease, rheumatoid arthritis) and cancers (endometrial, ovarian, lung), among others in 5 patients with MM.

These data suggest that each type of cancer or disease may have a characteristic signature in their thermogram (Garbett, et al., 2007, 2009; Michnik, 2011). This method is based on the biophysical technique of DSC, which monitors heat changes in a sample as a function of temperature. Analysis of plasma proteins using DSC is therefore based on an entirely different physical property than those of size, charge and chemical interactions that are utilized by the techniques of electrophoresis, mass spectrometry and immunochemistry, which have been mainstays of plasma protein analysis to-date.

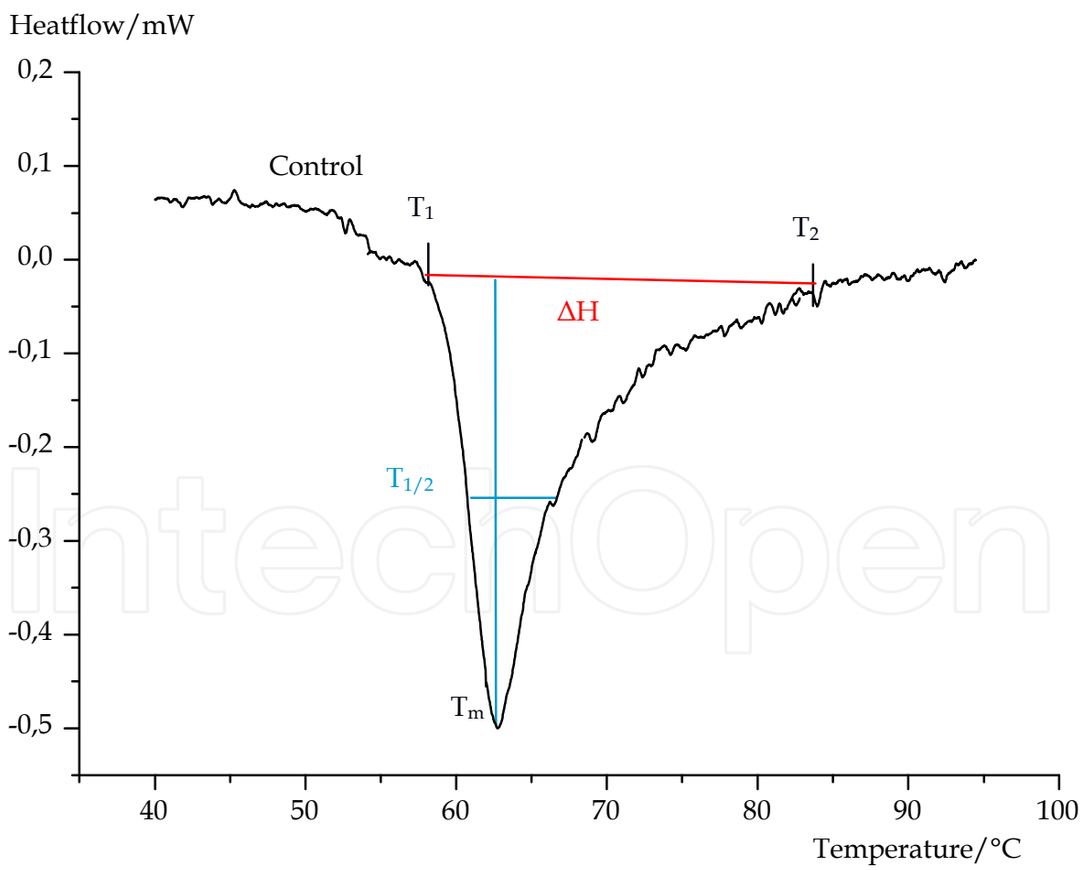

Fig. 1. DSC curve of human healthy control blood plasma
Transition temperatures: T_1: beginning of denaturation, T_2: end of denaturation;
Melting temperature: T_m; Calorimetric enthalpy: ΔH

This chapter will discuss the application of a DSC approach as a new diagnostic method for MM diagnosis and monitoring of patients with different clinical stages.

2.2.1 DSC examination of blood plasma of MM patients

In this study the primary melanoma and the sentinel lymph nodes painted with patent blue and labelled with ^{99m}Tc radiotracer were removed from 36 white adult patients (26 men and 10 women; median age, 61.3 years), who had histopathologically diagnosed operable MM. Surgery and follow-up were made in the Department of Dermatology, Venereology and Oncodermatology of the University Pecs, Hungary. MM was located on the head and neck (11%), on trunk (39%), on the upper limbs (27%), and on the lower limbs (23%). Regional lymph nodes were positive in 12 patients, while distant metastases (lung, brain) were diagnosed in 5 cases. From histopathological parameters tumour thickness were evaluated according to Breslow's, which is a prognostic factor of MM. This parameter changed from 0.5 mm to 8.3 mm in our patients. Clark's level is a related staging system, used in conjunction with Breslow's depth, which describes the level of anatomical invasion of the melanoma in the skin. Clark's level has prognostic significance only in patients with very thin (Breslow's depth <1 mm) melanomas. Invasion value was between Clark level II and IV in this study. The protocol was approved by regional ethical committee of Pecs University (27.06.2008/3220).

Peripheral blood samples of healthy controls (n=10) and preoperatively from MM patients (n=35) were collected and plasma components were analyzed by DSC technique. The thermal unfolding of the human plasma components were monitored by SETARAM Micro DSC II calorimeter. All experiments were conducted between 0 and 100 °C. The heating rate was 0.3 K/min in all cases. Conventional Hastelloy batch vessels were used during the denaturation experiments with 850µL sample volume in average. Reference sample was contained normal saline (0.9% NaCl). The sample and reference samples were equilibrated with a precision of ±0.1 mg. There was no need to do any correction from the point of view of heat capacity between sample and reference samples. The repeated scan of denatured sample was used as baseline reference, which was subtracted from the original DSC curve. Calorimetric enthalpy was calculated from the area under the heat absorption curve by using two-point setting SETARAM peak integration.

Comparison of DSC scans of healthy controls to the curves of cases with regional metastases, the DSC measurements showed 2 different thermal domains during the denaturation (Fig. 1., Fig. 2.). DSC measurements showed at least two marked different thermal domains during the denaturation. The first T_ms were only slightly influenced by the Breslow's depth and the Clark level (see Table 1 and 2), but it can be seen a difference in the melting enthalpies (Fig. 2.). The second T_ms and the calorimetric enthalpy changes demonstrated a significant difference of the melanoma depth dependence in 0.95-8 mm range and in Clark levels of II-IV (Fig. 2. as well as Table 1. and 2). These thermal parameters have been changed significantly in comparing with the control samples which were: T_ms 56 °C and 63 °C, ΔH~1.5 J/g. In the pathologic samples and in the progress of the decease one can separate a third thermal component between the first and second T_m (Fig. 2.). It is at around 62 °C, and it is shifted to higher temperature in case of regional metastasis. In cases of MM with distal metastases plasma denaturation exhibited an increase in second T_m, but a decrease in the calorimetric enthalpy (Fig. 2.).

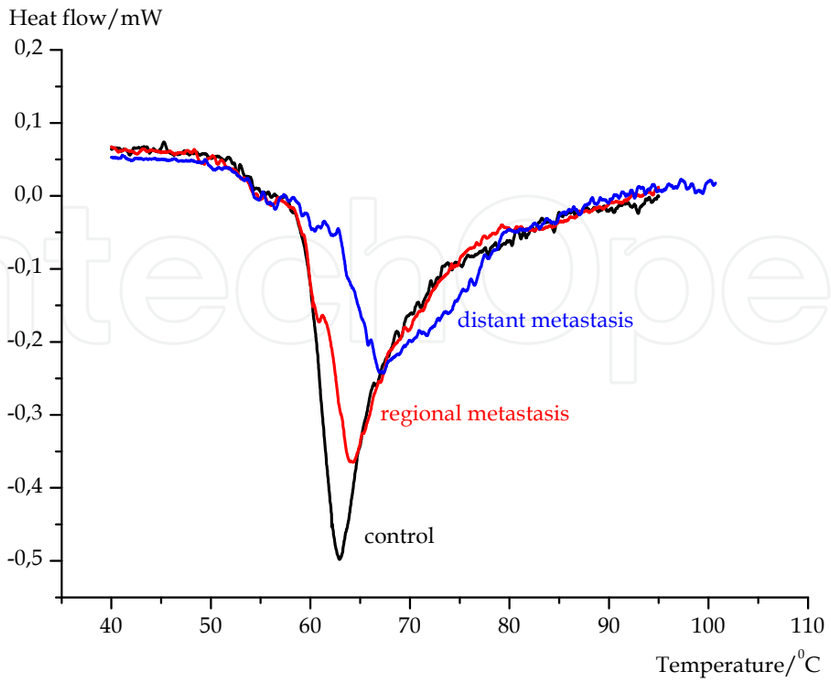

Fig. 2. DSC scans of human plasma in healthy controls, in MM patients with regional or distant metastases. Downward deflection represents endotherm process. (Black line: control, red line: MM with regional metastasis, blue line: MM with distant metastasis)

	T_{m1}/°C (mean±se)	T_{m2}/°C (mean±se)	ΔH(J/g) (mean±se)
Control (n=10)	56 ± 0.2	63.3 ± 0.3	1.53 ± 0.02
Breslow's depth (mm)			
0.5-1.0 (n=7)	55.8 ± 0.2	64.1 ± 0.3	1.13 ± 0.02
1.1-1.5 (n=2)	56.3 ± 0.3	63.5 ± 0.02	1.61 ± 0.01
1.6-2.0 (n=2)	55.75 ± 0.3	67.15 ± 0.4	1.3 ± 0.02
2.1-3.0 (n=5)	56.16 ± 0.2	62.18 ± 0.02	1.45 ± 0.03
3.1-4.0 (n=3)	55.8 ± 0.04	63.2 ± 0.2	1.37 ± 0.02
4.1-6.0 (n=2)	55.7 ± 0.2	63.9 ± 0.4	1.25 ± 0.01
6.1-8.5 (n=3)	55.8 ± 0.01	64.5 ± 0.1	1.23 ± 0.04

Table 1. DSC data according to Breslow's depth of melanoma

Clark level	T_{m1}/°C (mean±se)	T_{m2}/°C (mean±se)	ΔH(J/g) (mean±se)
I. (n=2)	55.2 ± 0.3	64.6 ± 0.1	1.14 ± 0.02
II. (n=1)	55.8 ± 0.03	63 ± 0.01	1.45 ± 0.04
III. (n=10)	55.7 ± 0.4	64.63 ± 0.2	1.286 ± 0.03
IV. (n=12)	56.05 ± 0.2	60.4 ± 0.02	1.43 ± 0.03

Table 2. DSC data according to Clark level of melanoma

3. Conclusion

Malignant melanoma is one of the most aggressive malignancies in human and is responsible for almost 60% of lethal skin tumours. Its incidence has been increasing in white population in the past two decades. Moreover, metastatic MM is an incurable disease with high mortality rate. Patients with metastatic disease have an average survival of <1 year. This high mortality rate is largely the result of the resistance to chemotherapy and radiotherapy (Palmieri, et al., 2009; Uong & Zon, 2010).

Although for most tumours, diameter is a powerful prognostic attribute, this is not so for melanomas, which can be very broad and yet have a good prognosis. This is because most melanomas have an extensive in situ or superficially invasive component that does not contribute to metastatic potential. It is therefore misleading and not recommended to report the breadth of a melanoma in a pathology diagnosis (Elder, 2011). The extent of penetration of melanoma cells into the skin was recognized as a better predictor of survival than breadth in a few early studies and was first codified into a powerful model in 1967 by Clark and co-workers. The disease was classified into intraepidermal (Clark level I), invades papillary dermis (Clark level II), fills papillary dermis (Clark level III), invades reticular dermis (Clark level IV), and invades subcutaneus fat (Clark level V) (Clark et al., 1969). Clark's level is a related staging system, used in conjunction with Breslow's depth, which describes the level of anatomical invasion of the melanoma in the skin. In 1970, Breslow described a simple system for measuring the thickness of melanomas. The Breslow thickness is determined by measuring from the top of the granular layer of the overlying skin, or from the base of an overlying ulcer, to the deepest invasive melanoma cell. Clark's level has prognostic significance only in patients with very thin (Breslow's depth <1 mm) melanomas.

Adequate resection of the specimens and sentinel lymph node biopsies are important factors in management of MM. But, there is no definite proof that longevity of patients is affected by routine laboratory tests (Bandarchi et al., 2010). New diagnostic methods in development and progression of MM could be helpful to identify the molecular profiles underlying aggressiveness, clinical behaviour, and response to therapy as well as to better classify the subsets of melanoma patients with different prognosis and/or clinical outcome. The human plasma proteome holds great promise as a convenient specimen for disease diagnosis and therapeutic monitoring. Moreover, blood samples may be easily obtained from patients by minimally invasive, safe procedure. The novel calorimetric assays are described that provides a new window through which to view the properties of the human plasma proteome (Anderson, N.L. & Anderson, N.G., 2002; Bruylants, et al., 2005; Ebert, et al., 2006).

This study investigated the thermal changes of human blood plasma components in melanoma patients with or without regional lymph node metastases by DSC. Overview 36 patients' thermograms, we observed their individual characteristics compare to healthy controls. Similar observations have been described by Garbett et al. in their important calorimetric studies, where demonstrated average thermograms for individuals diagnosed with various diseases (Lyme disease, rheumatoid arthritis) and cancers (endometrial, ovarian, lung), among others in 5 patients with MM. These data suggest that each type of cancer or disease may have a characteristic signature in their thermogram (Garbett, et al., 2009).

In the present study, DSC scan of healthy controls and the curves of MM cases showed 2 different thermal domains during the denaturation. Moreover, patients' thermograms are shifted towards higher denaturation temperatures. These changes were confirmed in the literature, where the average thermogram was obtained from duplicate DSC runs on samples from 100 healthy individuals and from 5 MM patients' sample also. Moreover, the disease thermograms are apparently localized in a higher temperature range. These unique appearances present a key utility of this technology as a diagnostic method (Garbett, et al., 2008).

Examination of DSC data in different clinical stages of MM patients should observed closed correlation with melanoma thickness and the extent of regional invasion. The first T_m was slightly influenced by the Breslow's depth and the Clark level (I. and III.). But, the second T_m and the calorimetric enthalpy changes demonstrated the melanoma depth dependence in 0.95-8 mm range and in Clark levels of II-IV. The surprising jump out of all thermal data in 1.1-2.0 mm Breslow's depth range as well as the opposite change in the tendency of T_{m2} and ΔH need further investigation with increased number of patients and with finer filtering. The same could be the conclusion in case of Clark level II. and IV.

These facts are important for many reasons: DSC measurement is suitable not only to clear skin cancer diagnosis, but to separate the different stages of MM patients and to monitor the actual stage of individual's disease. However, there are no data in the literature indicating the possible diagnostic and staging method of human blood plasma by DSC in MM patients. Similar findings have been described in another report, where applied the DSC method to investigate its utility for disease staging. Gynaecologic oncology samples analyzed by the method yielded progressively shifted thermograms charting the advance of cervical cancer from pre-invasive cervical lesions through each stage of invasive carcinoma. The distinction between normal and high-grade squamous intraepithelial lesions is significant and indicates the utility of the DSC method for the rapid screening of cervical cancer (Garbett, et al., 2009). The exact explanations of these results are not yet known. However, DSC analysis of plasma from diseased individuals revealed significant changes in the thermogram which are suggested to result not from changes in the concentration of the major plasma proteins but from interactions of small molecules or peptides with these proteins.

From clinical perspective, MM is generally considered to be a highly aggressive cancer, although a small subset of patients with metastatic melanoma has a relatively indolent disease course (Tsao, et al., 2004). Histologically, mitoses are frequently apparent in sections of melanoma tumours and staining for proliferative markers such as Ki67 is usually positive (Ohsie, et al., 2008). In the earliest descriptions of melanoma, the disease was regarded as a tumour mass that developed in the skin and was often surrounded by satellites at the time of presentation. A few studies recognized that more superficial melanomas have a better prognosis.

DSC thermograms of MM cases with distant metastases are shifted more towards higher denaturation temperatures. No other DSC data in the literature in MM patient with metastatic phase. But, there was a progressive shift of the thermogram to higher denaturation temperatures in cases of invasive cervical cancer compare to high-grade squamous intraepithelial lesions. The thermograms appear to be distinct from all the other diseases and unique for high-grade squamous intraepithelial lesions and invasive cervical cancer. The distinction between normal and high-grade squamous intraepithelial lesions is significant and indicates the utility of the DSC method for the rapid screening of cervical

cancer. A more detailed analysis of the data has revealed a progressively shifted thermogram for each stage of the disease (Garbett, et al., 2009).

In summary, this is the first report examined thermal changes by DSC on human blood plasma in MM patients with different clinical stages. Blood collection is a simple procedure and convenient to perform, and the DSC thermogram confirmed unique signature for human plasma components reflecting the normal, the pathomorphological changes and staging differences in melanoma patients. Further studies are needed to elucidate these relationships, but this preliminary study indicates great potential for the application of DSC as a clinical diagnostic tool, for example during disease grading and staging processes.

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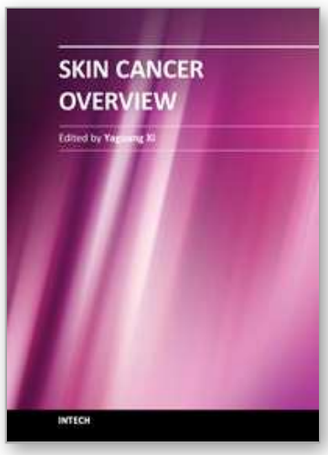

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The book Skin Cancer Overview is divided into three sections to cover the most essential topics in skin cancer research: Etiology, Diagnosis and Treatment, and Prevention. Due to the complexity of skin cancer, this book attempts to not only provide the basic knowledge, but also present the novel trends of skin cancer research. All chapters were written by experts from around the world. It will be a good handbook for researchers with interests in skin cancer.

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