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Reviews

Primary Scientific Studies

Case Reports


D
C
E
Fig. 7.
B
C
A
Fig. 6.
B
A
Fig. 8.
C
B
A
B
Fig. 2.
D
A
E
D
E
Fig. 9.
A

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Changing the Stage, Grade and Histological Subtypes of Renal Cell Carcinomas during 10 Years Period

Selahattin Çalışkan, Orhan Koca, Mehmet Akyüz, Metin İshak Öztürk, Muhammet Ihsan Karaman
Department of Urology, Haydarpasa Numune Training and Research Hospital, Istanbul, Turkey

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Key words: Renal cell carcinoma – Kidney – Carcinoma

Abstract: Renal cell carcinomas (RCCs) account 80–85% of all primary renal neoplasms and originate from the renal cortex. The patients who underwent radical or partial nephrectomy for renal tumour in our unit between January 2005 and 2015 were evaluated retrospectively. The patients were divided into two groups; group 1 includes patients who were treated between January 2005 and December 2009, group 2 those from January 2010 to 2015. There were 103 patients in group 1. The patients were between 21 and 89 years with mean age of 61.46 year. Renal cell carcinomas account 83.4% of the patients, benign renal tumours were 8.7% and transitional cell carcinomas were 7.7% of the patients in group 1. A total of 32.5% RCCs were classified as pT1a, 24.4% as pT1b, 15.1% as pT2a, 11.6% as pT2b, 15.1% as pT3a and 1.1% as pT4. There were 202 patients in group 2 and the patients were between 27 and 81 years with mean age of 58.5 year. Renal cell carcinomas comprised the main bulk of the tumours with 182 nephrectomy specimens. According to the pathological classification of RCCs, 51 specimens were found as pT1a, 54 were pT1b, 13 were pT2a, 14 were pT2b, 48 were pT3a and 2 were pT4. Although, the incidence of small renal masses has been increasing with widespread use of imaging techniques and recent advancements, the proportion of high grade and advanced stage renal tumours increased during the study period.

Mailing Address: Selahattin Çalışkan, MD., FEBU, Bahçelievler Mah. Çamlık Cad. No. 2 Çorum, Turkey; Phone: +905 547 846 552;
e-mail: dr.selahattincaliskan@gmail.com

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Introduction

Renal tumours are the 3rd most common cancer of all urologic tumours and account 3.5% of all malignancies (Çalışkan et al., 2014). Renal cell carcinomas originate from the renal cortex that accounts 80–85% of all primary renal neoplasms (Hashmi et al., 2014). Twenty-five to thirty percent of the patients with renal tumours are asymptomatic and are found on radiological examinations.

Table 1 – TNM classification of renal cell carcinoma

	Primary tumours (T)		
TX	primary tumour cannot be assessed		
T0	no evidence of primary tumour		
T1	tumour ≤ 7 cm in greatest dimension, limited to the kidney		
T1a	tumour ≤ 4 cm in greatest dimension, limited to the kidney		
T1b	tumour > 4 cm but ≤ 7 cm in greatest dimension, limited to the kidney		
T2	tumour > 7 cm in greatest dimension, limited to the kidney		
T2a	tumour > 7 cm but ≤ 10 cm in greatest dimension, limited to the kidney		
T2b	tumour > 10 cm, limited to the kidney		
T3	tumour extends into major veins or perinephric tissues but not into the ipsilateral adrenal gland and not beyond the Gerota fascia		
T3a	tumour grossly extends into the renal vein or its segmental (muscle-containing) branches, or tumour invades perirenal and/or renal sinus fat but not beyond the Gerota fascia		
T3b	tumour grossly extends into the vena cava below the diaphragm		
T3c	tumour grossly extends into the vena cava above the diaphragm or invades the wall of the vena cava		
T4	tumour invades beyond the Gerota fascia (including contiguous extension into the ipsilateral adrenal gland)		
	Regional lymph node (N)		
NX	regional lymph nodes cannot be assessed		
N0	no regional lymph node metastasis		
N1	metastasis in regional lymph node(s)		
N2	metastasis in more than 1 regional lymph node(s)		
	Distant metastasis (M)		
M0	no distant metastasis		
M1	distant metastasis		
	TNM stage grouping		
Stage I	T1	N0	M0
Stage II	T2	N0	M0
Stage III	T3	N0	M0
	T1, T2, T3	N1	M0
Stage IV	T4	any N	M0
	any T	N2	M0
	any T	any N	M1

There has been an increasing incidence of renal cell carcinomas, as a result of more prevalent use of imaging techniques, such as ultrasonography, computed tomography and magnetic resonance imaging in recent years (Nason et al., 2014).

Ultrasonographic examination is the first imaging technique for the patients to evaluate renal cell carcinomas (Sacco et al., 2011). Ultrasonography gives information on the size, attenuation features and vascular distribution of renal masses and adds useful diagnostic information to other imaging techniques. Computed tomography is the gold standard for diagnosis, staging, and surveillance of renal cell carcinomas. Magnetic resonance imaging is usually used for patients with contrast allergy, functional renal impairment and pregnancy.

We evaluated the clinico-pathological results of the patients with renal tumours during the last 10 years period.

Material and Methods

A retrospective data on the radical or partial nephrectomy for renal tumour performed in our unit from January 2005 to January 2015 was evaluated. Patient's demographics, tumour size, TNM classification, stage and histopathology data were recorded. TNM classification is used for renal cell carcinoma (Table 1). All nephrectomy specimens were analyzed in the same pathology department. The patients were divided into two groups; group 1 includes patients who were treated between January 2005 and December 2009, group 2 those from January 2010 to January 2015. Nephron-sparing surgery was performed for lesions that are peripheral, less than 7 cm in size and appropriate for resection. Radical nephrectomy was performed in other cases. All patients underwent nephrectomy with open surgical techniques.

Results

Among the 325 total patients with renal tumours that were treated surgically at our clinic were included in this study. The cases increased in number progressively from 103 to 202 during the ten years period. The clinicopathological parameters of each group are shown in Table 2.

There were 103 patients in group 1. The patients were between 21 and 89 years with a mean age of 61.46. The male patients were predominant with 66.3% of the patients and the female patients were 33.7% of the patients. Renal cell carcinomas account 83.4% of the patients, benign renal tumours were 8.7% and transitional cell carcinomas were 7.7% of the patients. The histological subtypes of renal cell carcinomas (RCCs); 64 (74.4%) were clear cell, 12 (13.9%) were chromophobe, 9 (10.4%) were papillary and 1 (1.5%) was multiloculer cystic RCC. A total of 28 (32.5%) RCCs were classified as pT1a, 21 (24.4%) as pT1b, 13 (15.1%) as pT2a, 10 (11.6%) as pT2b, 13 (15.1%) as pT3a and 1 (1.1%) as pT4. Open nephron-sparing surgery were performed for 12 (11.6%) patients and the other patients were treated with open radical nephrectomy.

Table 2 – Patient demographics and tumour characteristics of the groups

	Group 1	Group 2
Total number	103	202
Gender		
Male, n (%)	68 (66.3)	142 (70.3)
Female, n (%)	35 (33.7)	60 (29.7)
Tumour size (cm)		
Mean (±SD)	6.66 ± 3.65	6.3 ± 3.5
Age and decades, n (%)		
Mean age (±SD)	61.46 ± 13.71	58.5 ± 11.22
<30	1 (0.9)	2 (1)
30–39	7 (6.7)	10 (5)
40–49	13 (12.6)	37 (18.3)
50–59	23 (22.3)	56 (27.7)
60–69	27 (26.2)	61 (30.1)
70–79	24 (23.3)	34 (16.8)
80–89	8 (7.7)	2 (1)
Procedure		
Partial, n (%)	12 (11.6)	47 (23)
Radical, n (%)	91 (88.3)	157 (77)
Pathological examination		
Renal cell carcinoma	86 (83.4)	182 (89.2)
Transitional urothelial carcinoma	8 (7.7)	6 (2.9)
Benign renal tumours	9 (8.7)	14 (6.9)
Others		2 (1)

SD – standard deviation

There were 202 patients in group 2, 142 (70.3%) of the patients were male, 60 (29.7%) of the patients were female. The patients were between 27 and 81 years with the mean age of 58.5. Two patients had bilateral renal tumours and the number of the specimen was increased to 204. Renal cell carcinomas comprised the main bulk of the tumours with 182 (89.2%) nephrectomy specimens. Of these tumours, 129 (70.9%) cases were clear cell, 21 (11.5%) were chromophobe, 21 (11.5%) were papillary, 7 (3.4%) were unclassified and there were one cases (0.5%) with renal medullary carcinoma, mucinous tubular and spindle cell carcinoma, multilocular cystic and oncocytoid RCCs. According to the pathological classification of RCCs, 51 specimens were found as pT1a, 54 were pT1b, 13 were pT2a, 14 were pT2b, 48 were pT3a and 2 were pT4. Comparison of TNM stage, histological subtype and nuclear grade of RCCs are shown in Table 3. Other malignant tumours; transitional urothelial carcinoma was reported in six patients,

Table 3 – Pathological examination of the patients with renal cell carcinomas

	Group 1	Group 2
Total, n	86	180
Male, n (%)	54 (62.7)	127 (70.6)
Female, n (%)	32 (37.2)	53 (29.4)
Pathologic T stage, n (%)		
pT1a	28 (32.5)	51 (28)
pT1b	21 (24.4)	54 (29.7)
pT2a	13 (15.1)	13 (7.1)
pT2b	10 (11.6)	14 (7.7)
pT3a	13 (15.1)	48 (26.3)
pT3b	0 (0)	0 (0)
pT4	1 (1.1)	2 (1)
N stage, n (%)		
N0	84 (97.6)	173 (95)
N1	2 (2.4)	0
N2	0	9 (5)
M stage, n (%)		
M0	86 (100)	178 (98.9)
M1	0	2 (1.1)
Stage, n (%)		
I	49 (57)	103 (56.5)
II	23 (26.7)	24 (13.2)
III	11 (12.8)	43 (23.6)
IV	3 (3.4)	12 (6.6)
Histological subtype, n (%)		
Clear cell	64 (74.4)	129 (70.9)
Chromophobe	12 (13.9)	21 (11.5)
Papillary	9 (10.4)	21 (11.5)
Multilocular cystic	1 (1.5)	1 (0.5)
Unclassified	0	7 (3.4)
Others	0	3 (1.5)
Nuclear grade, n (%)	83 (100)	175 (100)
G1	8 (9.6)	11 (6.2)
G2	39 (47)	75 (43)
G3	26 (31.3)	72 (41.1)
G4	10 (12)	17 (9.7)

squamous cell carcinoma and rhabdomyosarcoma was diagnosed in one case in group 2. Open nephron-sparing surgery was performed for 47 (23%) tumours and open radical nephrectomy was done for 157 (77%) tumours.

Discussion

Although renal cell carcinoma (RCC) represents 2–3% of all adult malignant neoplasms, it is the most lethal urologic cancer (Pantuck et al., 2000). More than 40% of the patients with RCC have died of their cancer; only 20% of mortality rate is associated with bladder and prostate cancers (Gupta et al., 2010). The incidence of small renal masses has been increased with the widespread use of imaging techniques and recent advancements (Akgül and Tinay, 2013). Nephron-sparing surgery (NSS) has been accepted as a gold standard treatment for small renal tumours, because of its comparable oncological outcome, improved survival and decreased renal insufficiency when compared with radical nephrectomy (Demirer and Yıldırım, 2013). There are some types of NSS techniques; laparoscopic, robotic or open. Open partial nephrectomy is the standard treatment for T1 tumours according to the EAU (European Association of Urology) guidelines (Ljungberg et al., 2013). The renal tumour cases were increased 96.1% and NSS was done nearly two times more in the last 5 years. The incidence of RCCs in renal tumour cases was increased in last 5 years in our study.

Patients with RCCs are usually older, with the mean age at diagnosis of 60 years (Ozbek et al., 2013). The highest incidence of this disease is found in North America and Europe. Although, the etiology of RCC is unclear, smoking, obesity, antihypertensive therapy, coffee, tea, Western diet (high in fat and protein, low vegetables and fruits) are risk factors (Chen et al., 2009). In a study from Austria, the authors found that, the median age of the patients were between 61 and 65 over the 27 years (Pichler et al., 2012). We found the mean age of the patients was 61.46 and 58.5 in the first and second 5 years.

Renal cell carcinoma is almost twice as common in men as in women (Chen et al., 2009). The male/female ratio is 1 in the population <40 years. The risk of RCC will be higher in young women; the studies reported relationship between RCC and some reproductive and hormonal factors such as high corporal hormone levels of young patients. The male/female ratio may be different in the studies. While this ratio was 1.9 in a study from China (Chen et al., 2009), was 1.39 in a study from Austria (Pichler et al., 2012). We found the male/female ratio was 2.13 in the patients with RCCs.

The tumour subtypes of RCCs have been published in 2004 by the World Health Organization (Dutcher, 2013). The most common subtype of RCC is clear cell that comprises 75% of all RCCs in surgical series (Cheville et al., 2003). Papillary RCCs account 15% of surgical series and chromophobe RCCs are about 5% of all RCCs. Clear cell RCCs was the most common subtype of RCCs followed by chromophope RCCs in our study. Our study found that papillary RCCs were the third most common subtype in two groups. Multilocular cystic RCC is a rare form of clear cell RCC and has a good survival (Çalışkan et al., 2011). There was only one case in the groups.

The tumour, nodes and metastasis stage classification system is generally recommended for clinical use and has introduced significant changes based on recent literature in 2010 (Table 3) (Ljungberg et al., 2013). The patients with organ confined tumours (pT1 and pT2) have decreased from 83.6 to 72.5%, in the contrary, pT1b tumours were increased from 24.4 to 29.7% in our study. The authors reported that the incidence of organ confined tumour was increased from 36.9 to 73.2% during the 27 years (Pichler et al., 2012); the other study demonstrated that the percentage of organ confined tumours was 66.7, 56.2 and 65.6% in the three study group respectively (Nason et al., 2014). The patients with local advanced tumours (pT3 and pT4) have increased during the study period (from 16.2 to 27.3%). The authors observed a decrease in pathological pT3 and pT4 RCC patients from 29.1 to 20.5% over two decades (Gupta et al., 2010). The patients with local advanced RCC have increased during the seventeen years period (from 33.3 to 34.4%) in another study (Nason et al., 2014). One of the reasons of increasing numbers of patients with local advanced tumour: our hospital is tertiary referral centre. The other factor may be the indications of nephrectomy that has been changed during the years with surgical experience.

Fuhrman nuclear grade (based on nuclear size and shape and the prominence of nucleoli) is a prognostic factor in RCC (Gupta et al., 2010). Higher grade is associated with the biological aggressiveness and increased metastatic potential of the tumour. Most of the RCCs are classified as grade II in the studies (Pichler et al., 2012; Ozbek et al., 2013). The proportion of grade II tumours was between 43 and 61.5%. There is an increasing representation of grade III and IV tumours over time, from 12.7 to 20.3% and decreasing the proportion of grade I tumours from 32 to 14.8% in an analysis of 2,739 patients (Pichler et al., 2012). Additionally, the authors reported that there was a significant increase of grade III tumours over time, from 17.6 to 30.8% and decreasing proportion of grade I tumours from 16.2 to 7.1% (Doeuk et al., 2010). On the contrary, grade III and IV tumours were decreased from 30.4 to 10.1% and from 8.5 to 4.2% in another study from India (Gupta et al., 2010). We found that most of the patients with RCC were grade II; grade III tumours were increased over the study period from 31.3 to 41.1%.

The authors found an increasing representation of stage I tumours from 51 to 60%, decreasing proportions of stage II and III diseases in a large retrospective American study (Kane et al., 2008). The pathological stage II and III were decreased from 14.5 to 11.6% and from 22.8 to 16.3%. The pathological stage IV remained stable over the 12 year period at about 12%. The investigators reported that the pathological stage III were increased from 13.9 to 21.5%, stage II and IV were decreased from 18.1 to 11.1% and from 1.4 to 0.4% during the 15 years (Doeuk et al., 2010). The proportion of the pathological stage I was constant throughout the 15 year period with 67%. We found that the proportions of pathological stage I tumours were similar in groups and stage II tumours were decreased from 26.7 to

13.2%. The pathological stage III and IV diseases were increased from 12.8 to 23.6% and from 3.4 to 6.6% in our study.

The proportion of benign renal tumours has decreased over time (from 8.7 to 6.9%). One of the factors may be the result of the developing techniques of radiological imaging. The widespread use of cross sectional imaging has led to an increasing diagnosis of incidental and small renal lesions (Satasivam et al., 2012). Optimal management is controversial, with available options including surveillance, ablation and surgical resections. The patients may underwent surveillance or ablation therapies instead of surgery.

Renal pelvis tumours are 5–7% of all renal tumours (Ozsahin et al., 2009). The most common type is transitional cell carcinomas. Squamous cell carcinomas, adenocancers and sarcomas are rare forms. Radical surgery is essential for transitional cell carcinomas. While the incidence of these tumours was similar in group 1, less than literature in group 2.

There are several limitations in the present study. First, the study does not include very large numbers of patients and our databases includes only pathologically diagnosed renal tumours, the patients that have been treated with minimally invasive ablation or surveillance were not included. The patients underwent surgical treatment by multiple surgeons and pathological specimens of the patients were examined different pathologist in pathology department. Despite these limitations, the present study is the largest single centre database analysis of kidney cancer stage, grade and histological changes over one decade in Turkey.

In conclusion, the percentage of partial nephrectomy has increased. The proportion of the patients with higher grades, local advanced renal cell carcinomas and stage III and IV increased during the study period. Although extensive use of imaging, epidemiologic studies are needed to define the results.

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 Prague Medical Report / Vol. 118 (2017) No. 4, p. 128–138

5-fluorouracil Toxicity Mechanism Determination in Human Keratinocytes: *in vitro* Study on HaCaT Cell Line

Jan Hartinger[1], Pavel Veselý[2], Martin Šíma[1], Irena Netíková[1], Eva Matoušková[3], Luboš Petruželka[4]

[1]Department of Clinical Pharmacology and Pharmacy, Institute of Pharmacology, First Faculty of Medicine, Charles University and General University Hospital in Prague, Prague, Czech Republic;
[2]Central European Institute of Technology, Brno University of Technology, Brno, Czech Republic;
[3]Department of Burns Medicine, Third Faculty of Medicine, Charles University and University Hospital Královské Vinohrady, Prague, Czech Republic;
[4]Department of Oncology, First Faculty of Medicine, Charles University and General University Hospital in Prague, Prague, Czech Republic

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Abstract: 5-fluorouracil (5-FU) and capecitabine therapy is often accompanied by palmar-plantar erythrodysesthesia (PPE) which is manifestation of 5-FU toxicity in keratinocytes. The main mechanisms of 5-FU action are thymidylate synthase (TS) inhibition which can be abrogated by thymidine and strengthened by calciumfolinate (CF) and incorporation of fluorouridinetriphosphate into RNA which can be abrogated by uridine. For proper PPE treatment 5-FU mechanism of action in keratinocytes needs to be elucidated. We used the 5-FU toxicity modulators uridine, thymidine and CF to discover the mechanism of 5-FU action in human keratinocyte cell line HaCaT. To measure the cellular viability, we used MTT test and RTCA test. CF did not augment 5-FU toxicity and 5-FU toxicity was weakened by uridine. Therefore, the primary mechanism of 5-FU toxicity in keratinocytes is 5-FU incorporation into RNA. The uridine protective effect

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Mailing Address: Mgr. Jan Hartinger, Department of Clinical Pharmacology and Pharmacy, Institute of Pharmacology, First Faculty of Medicine, Charles University and General University Hospital in Prague, Na Bojišti 1, 128 00 Prague 2, Czech Republic; Phone: +420 224 964 135; e-mail: hartinger.jan@vfn.cz

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cannot fully develop in the presence of CF. Thymidine addition to 5-FU and uridine treated cells not only prevents the toxicity-augmenting CF effect but it also prolongs the 5-FU treated cells survival in comparison to uridine only. Therefore, it can be assumed that in the presence of uridine the 5-FU toxicity mechanism is switched from RNA incorporation to TS inhibition. Although particular 5-FU toxicity mechanisms were previously described in various cell types, this is the first time when various combinations of pyrimidine nucleosides and CF were used for 5-FU toxicity mechanism elucidation in human keratinocytes. We suggest that for PPE treatment ointment containing uridine and thymidine should be further clinically tested.

Introduction

5-fluorouracil (5-FU) and its peroral prodrug capecitabine are the cornerstone cytostatics in the treatment of various tumours especially colorectal carcinoma (Longley et al., 2003; Engstrom et al., 2009; Schmoll et al., 2012). Palmar-plantar erythrodysesthesia (PPE) is 5-FU adverse event that occurs when 5-FU is applied as continuous infusion (Lokich et al., 1989; Wolpin and Mayer, 2008) and accompanies also the long-term capecitabine peroral therapy in as much as 60% of patients (Leonard et al., 2011; Hofheinz et al., 2012). This makes PPE one of the most frequent capecitabine adverse events (Biganzoli et al., 2002; Yun et al., 2010; Leonard et al., 2011). PPE is caused by keratinocyte reaction to the cytotoxic compound presented in the skin (Janusch et al., 2006). So far no satisfactory PPE treatment is available. One of the treatment approaches is local 5-FU antidote application which does not influence systemic 5-FU efficacy (Barth, 2004; Netíková et al., 2009). Nowadays no ideal locally applied antidote becomes widely recognized as effective. Pharmacy compounded 10% uridine ointment is used as empirical clinical praxis in several oncology centers in Europe (Barth, 2004; Netíková et al., 2009). No randomized controlled trials support this treatment and placebo effect or ointment base effect cannot be ruled out. Different 5-FU toxicity mechanism occurs in different cell types (Umeda and Heidelberger, 1968; Martin et al., 1980; van Groeningen et al., 1992). The particular mechanism of 5-FU toxicity in keratinocytes needs to be elucidated for locally applicable PPE antidote development. Therefore, we aimed to find out the particular 5-FU toxicity mechanism in these cells to help the best local antidote ointment development.

Two main biological mechanisms of 5-FU action are *thymidylate synthase (TS) inhibition by fluorodeoxyuridinmonophosphate (FdUMP)* and *fluorouridintriphosphate (FUTP) incorporation into RNA*. FdUMP-TS complex is further stabilized by reduced folic acid which normally serves as methyl donor for uridinemonophosphate (UMP) to thymidylate (TMP) conversion. Consequently, high concentration of dUTP occurs in the cell which leads to dUTP incorporation into DNA and subsequent DNA damage (Martin et al., 1980; Longley et al., 2003). TS inhibition can be abrogated by adding of thymidine, which is metabolized by thymidinkinase to

thymidylate without contribution of TS (Umeda and Heidelberger, 1968). Therefore, in the presence of thymidine the 5-FU effect on DNA is weakened which leads to more pronounced effect on RNA mediated by fluorouridintriphosphate (FUTP) incorporation into RNA and subsequent RNA function disruption (Martin et al., 1980; Longley et al., 2003) which is preventable by uridine (Sawyer et al., 1984; Codacci-Pisanelli et al., 2008).

The 5-FU toxicity modulators can be used for studying the particular 5-FU toxicity mechanism in particular cell line (Umeda and Heidelberger, 1968; Codacci-Pisanelli et al., 2008). If 5-FU toxicity is strengthened by calciumfolinate (CF) addition, the TS inhibition plays significant role whereas if the 5-FU toxicity is not augmented by CF, RNA toxicity is main mechanism of 5-FU action. If the toxicity decreases after uridine addition, the 5-FU incorporation into RNA plays important role (Codacci-Pisanelli et al., 2008). If the toxicity is lowered after thymidine addition which abrogates TS inhibition, DNA toxicity is the major mechanism of 5-FU action (Umeda and Heidelberger, 1968) whereas opposite effect of thymidine addition means that augmentation of the 5-FU incorporation to RNA results in more toxic effect than TS inhibition (Martin et al., 1980). Here we studied the 5-FU mechanism of action in human spontaneously immortalized HaCaT cell line by adding various combinations of uridine, thymidine and CF to the cell culture medium.

Material and Methods

Cultivation of HaCaT keratinocytes

HaCaT cells were kindly provided by professor Dr. J. Bereiter-Hahn, Kinematic Cell Research Group, Institute for Cell Biology and Neurosciences, Goethe University Frankfurt am Main, Germany. HaCaT cells are a spontaneously immortalized human epithelial cell line that maintains the full epidermal differentiation capacity (Boukamp et al., 1988). The cells were cultured in the H-MEM medium supplemented with non-essential amino acids, 0.12 g/l sodium pyruvate, 1 g/l $NaHCO_3$, 10% bovine serum, 2% fetal bovine serum and antibiotics (200 U/ml penicillin and 100 µg/ml streptomycin). The cells were maintained in the humidified atmosphere at 37 °C and 3.5% CO_2.

MTT test

We tested the effect of uridine, thymidine and CF on cells treated with various 5-FU concentrations. We used the MTT test described elsewhere (Mosmann, 1983). The cells were plated into 96-well plate (14 000 cells per well in 100 µl of the medium without phenol red). The outer wells were not seeded with cells but filled with sterile water for injection. When cellular layers were almost confluent, 100 µl of the tested agents dissolved in the medium were added to the wells. For each of the protective substances settings (5-FU various concentrations, nucleosides and CF combinations) 9 wells were used. The metabolic activity

was measured after 2, 4 and 7 days of cultivation. 10 µl of MTT (5 mg/ml in PBS) was added into three of the wells with each particular compound combination. After six hours of incubation the formazan production was stopped by 100 µl of the 10% SDS solution in distilled water. After overnight incubation the plates were analyzed by the ELISA reader (570 nm test wavelength and the 630 nm background wavelength). The mean values of absorbance from the wells with the same concentration of the tested agents (3 for each setting) were considered as indicators of the cellular metabolic activity. The higher absorbance was measured the more viability of the cells.

RTCA (xCELLigence)

We tested the effect of uridine, thymidine and CF on cells treated with 7.5 µg/ml 5-FU concentration. We used the Real Time Cell Analyser produced by Roche Applied Sciences (xCELLigence). Fourteen thousand cells in the 100 µl medium were plated into the wells of a 96-well plate. Each well is provided with golden electrodes on the bottom surface. The measurable impedance between these two electrodes increases when the cells are growing and dividing. The cell surface changes and the adhesion and morphology changes play a role in this measurement, too. As a result, we obtain the "cell index" derived from the above-mentioned cellular properties. The cell index can be generally considered as the cellular viability indicator with some limitations (Atienzar et al., 2011). The outer wells were not used as the cells in these wells are frequently affected by evaporation. The tested substances diluted in 100 µl of the medium were added when the cell index plot curves were growing exponentially. The cell index value was recorded every 3.5 hours for 6 days. In the plot the mean value of cell index derived from 3 wells for each tested compounds combination is presented. Time 0 represents the time of adding the tested compounds.

The tested substances

Uridine and thymidine were obtained as > 99% powder in cell-culture suitable quality from Sigma-Aldrich (SIGMA-ALDRICH s. r. o., Prague, Czech Republic). 5-FU was obtained as a 50 mg/ml solution in water for injection and calciumfolinate was obtained as a 10 mg/ml solution in water for injection from local hospital pharmacy (both from EBEWE Pharma Ges. m. b. H. Nfg. KG, Unterach, Austria). All tested substances were further diluted in the cell culture medium without addition of any solvent. The calciumfolinate was tested in 20 µg/ml concentration. Uridine was tested in 50 µg/ml and thymidine was tested in 25 µg/ml concentration. 5-FU was tested in 4.8, 7.6, 12.2, 9.5 and 31.3 µg/ml concentrations.

Statistics

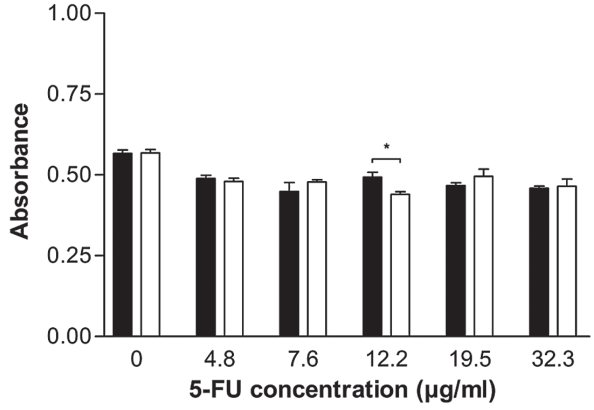
To compare the MTT test cellular viability results and RTCA cell index differences we used student's *t*-test.

Results

The metabolic activity and surface adherence of keratinocytes did not differ significantly when calciumfolinate was added to the 5-FU treated cells (Figures 1A and B and 4A). The 5-FU toxicity was directly proportional to the 5-FU concentration on day 4 (Figure 1B). On day 7 all cells without nucleosides were already dead because of 5-FU toxicity in MTT test (results not shown) and in RTCA test (Figure 4A).

Uridine addition lowered 5-FU toxicity in HaCaT cells (Figures 2 and 4B). The toxicity of 5-FU was pronounced to the similar extent on the day 4 irrespectively on the 5-FU concentration when uridine without CF was added (Figure 2B). When CF was added to the 5-FU-uridine combination, the protective effect of uridine was weakened and the toxicity of 5-FU was significantly augmented (Figures 2

A. Day 2: 5-FU only and 5-FU + calciumfolinate



B. Day 4: 5-FU only and 5-FU + calciumfolinate

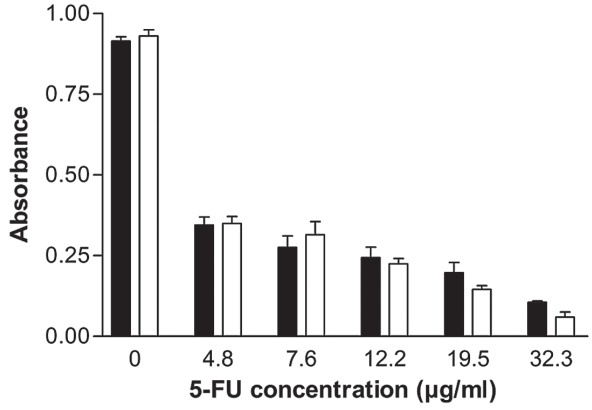
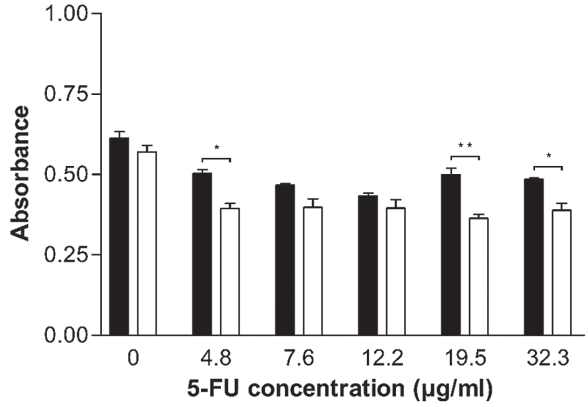


*Figure 1 – Metabolic activity of HaCaT cells measured by MTT test. The higher the absorbance is the higher the metabolic activity. Comparison of cells treated with 5-FU only (black columns) and with 5-FU together with 20 µg/ml calciumfolinate (white columns). Calciumfolinate addition to 5-FU treated cells did not augment the 5-FU toxicity in days 2 (A) and 4 (B) (*p<0.05).*

A. Day 2: Uridine protective effect against 5-FU toxicity is lowered by calciumfolinate addition



B. Day 4: Uridine protective effect against 5-FU toxicity is lowered by calciumfolinate addition

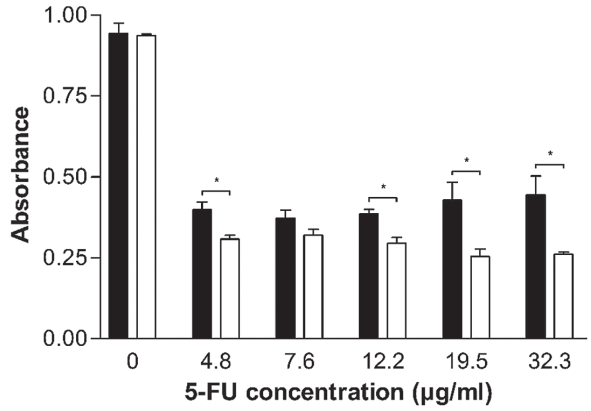


*Figure 2 – Metabolic activity of HaCaT cells measured by MTT test. The higher the absorbance is the higher the metabolic activity. Comparison of cells treated with 5-FU + uridine (50 µg/ml) without CF (black columns) and cells treated with 5-FU + uridine (50 µg/ml) with 20 µg/ml calciumfolinate (white columns). Calciumfolinate addition to 5-FU + uridine treated cells augmented the 5-FU toxicity in days 2 (A) and 4 (B) (*p<0.05, **p<0.01).*

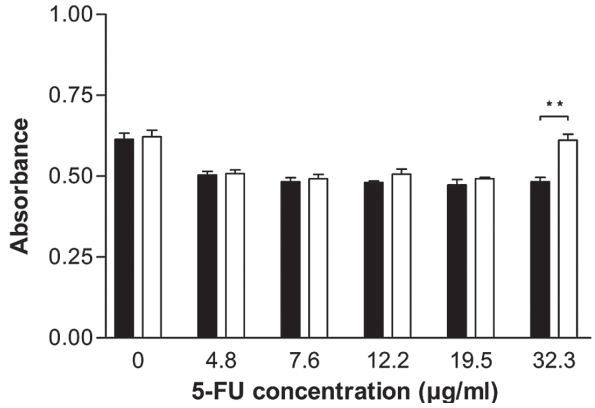
and 4B). On day 4 the CF effect was significantly more pronounced in higher 5-FU concentrations (Figure 2B). On day 7 all cells were already dead in MTT (results not shown) and RTCA test (Figure 4B).

When uridine, together with thymidine were added to the cells treated with 5-FU CF did not augment the 5-FU toxicity and most of the cells survived till day 7 in MTT (Figure 3C) and RTCA (Figure 4C) test. On day 7 the 5-FU toxicity was more pronounced in higher 5-FU concentrations in MTT test (Figure 3C).

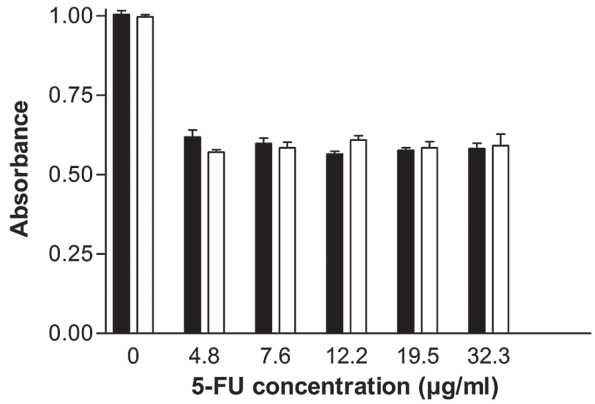
Discussion

Although particular 5-FU toxicity mechanism were previously described for various cell types (Codacci-Pisanelli et al., 2008), this is the first time when

A. Day 2: Uridine and thymidine protective effect is not lowered by calciumfolinate addition



B. Day 4: Uridine and thymidine protective effect is not lowered by calciumfolinate addition



C. Day 7: Uridine and thymidine protective effect is not lowered by calciumfolinate addition

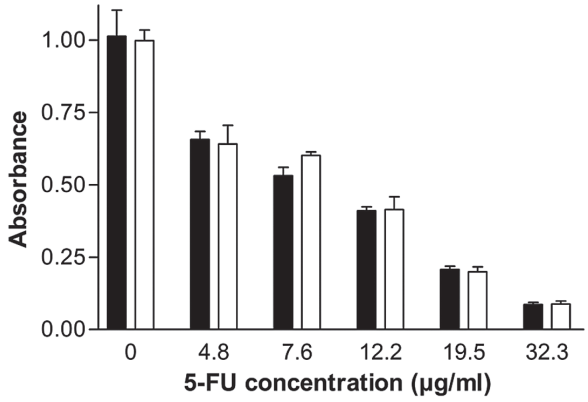


*Figure 3 – Metabolic activity of HaCaT cells measured by MTT test. The higher the absorbance is the higher the metabolic activity. Comparison of cells treated with 5-FU + uridine (50 µg/ml) + thymidine (25 µg/ml) without CF (black columns) and cells treated with 5-FU + uridine (50 µg/ml) + thymidine (25 µg/ml) with 20 µg/ml calciumfolinate (white columns). Calciumfolinate addition to the 5-FU treated cells did not augment the 5-FU toxicity in the presence of uridine and thymidine combination in days 2 (A), 4 (B) and 7 (C) (**p<0.01).*

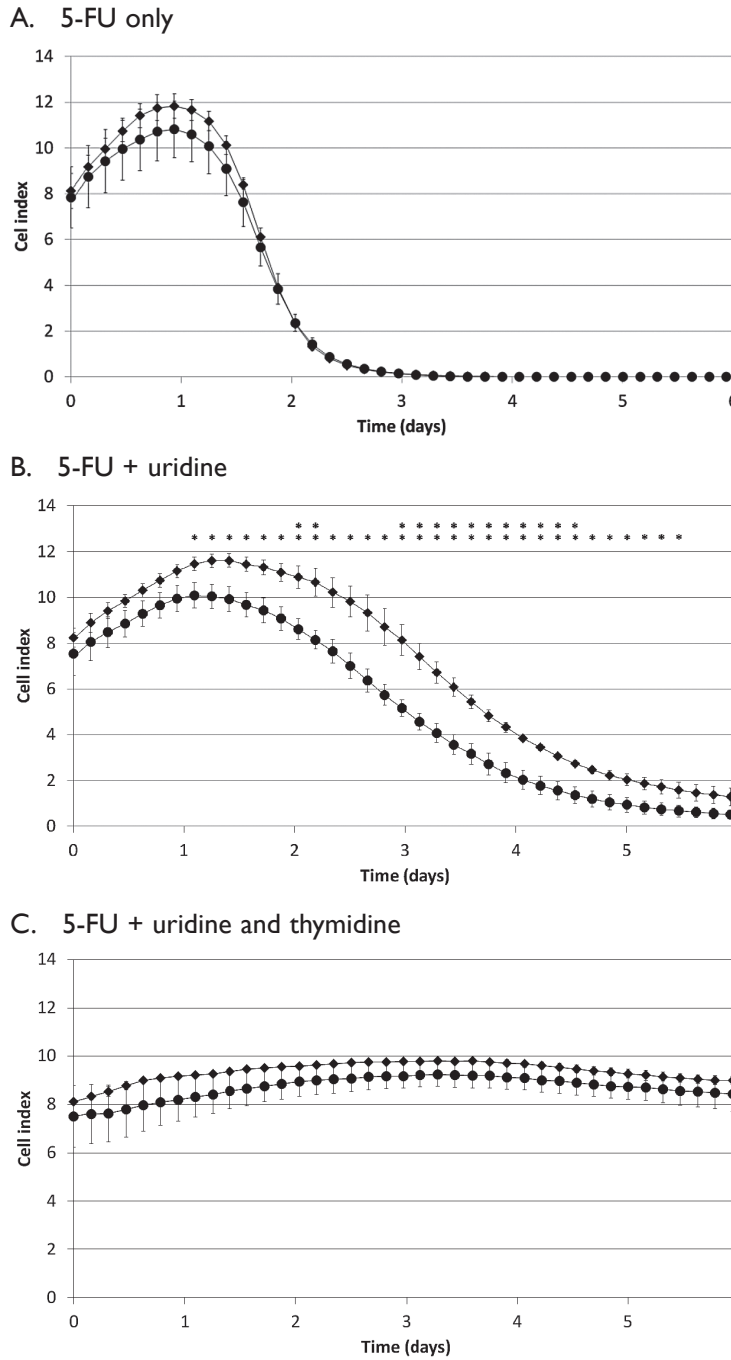


*Figure 4 – Effect of calciumfolinate addition to the 5-FU (7.5 μg/ml) treated cells (A), 5-FU (7.5 μg/ml) + uridine (50 μg/ml) treated cells (B) and 5-FU (7.5 μg/ml) + uridine (50 μg/ml) and thymidine (25 μg/ml) treated cells (C). Diamonds – cells without calciumfolinate, circles – cells with 20 μg/ml calciumfolinate (*p<0.05, **p<0.01).*

various combinations of pyrimidine nucleosides and CF were used for 5-FU toxicity mechanism elucidation in human keratinocytes. We confirmed that CF did not augment the 5-FU toxicity in keratinocytes (Figures 1A and B and 4A). Similarly as in other non-tumorous tissues (Sawyer et al., 1984; Bagrij et al., 1993; Codacci-Pisanelli et al., 1997, 2008; Pritchard et al., 1997) the primary mechanism of 5-FU action in keratinocytes is therefore mediated by its incorporation into RNA. We have shown that this primary mechanism of 5-FU toxicity in HaCaT keratinocytes is preventable by uridine (Figure 2A and B). Our results also show that uridine addition "switches" the toxicity mechanism from 5-FU RNA incorporation to TS inhibition because CF strengthened the 5-FU toxicity when added to the 5-FU + uridine treated cells (Figures 2A and B and 4B). When thymidine is added to the 5-FU + uridine treated cells both of the two main 5-FU toxicity mechanisms are prevented and the cells survive significantly longer than those treated by uridine only (Figures 3C and 4C). CF did not augment the 5-FU toxicity when thymidine was added to the cell medium because of the TS inhibition was abrogated (Figures 3A–C and 4C). In the presence of uridine and thymidine combination the toxicity of 5-FU was more pronounced in higher concentrations of 5-FU on day 7 (Figure 3C). Because of the aforementioned absence of CF effect this toxicity cannot be adjudged to TS inhibition. When both main 5-FU toxicity mechanisms are abrogated by uridine and thymidine, other minor toxicity mechanisms may become important (Bagrij et al., 1993; Wurzer et al., 1994; An et al., 2007; Yen-Revollo et al., 2008).

5-FU DNA incorporation (An et al., 2007) may became important in higher 5-FU concentrations. Because 5-FU DNA incorporation would be at least partially preventable in the surplus of thymidine, lower 5-FU concentration toxicity would be prevented but higher 5-FU concentrations toxicity would be preserved. 5-FU degradation product toxicity (Yen-Revollo et al., 2008) or unmetabolized 5-FU toxicity (Bagrij et al., 1993; Wurzer et al., 1994) may also play role in higher 5-FU concentrations.

Conclusion

Our results show that not only the cell type and state (e. g. frequency of mitoses and amount of DNA synthesis) but also its environment e. g. amount of nucleosides in the cell surrounding predisposes the cells for particular 5-FU toxicity mechanism development. In human keratinocytes RNA toxicity preventable by uridine is the primary mechanism of 5-FU action. In the presence of uridine TS inhibition becomes more important. This can be further abrogated by thymidine. In the development of local antidote ointment in PPE treatment it is favourable to antagonize most of the probable 5-FU toxicity mechanisms. Therefore, our findings should be considered when developing the most potent local antidote ointment for 5-FU induced skin adverse events.

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Catastrophic Left Ventricular Thrombosis Complicating Extra-corporeal Membrane Oxygenation: A Case Report

Michal Pořízka[1], Petr Kopecký[1], Vladimír Mikulenka[2], Jan Kunstýř[1], Michal Lipš[1], Martin Balík[1]

[1]Department of Anaesthesiology, Resuscitation and Intensive Medicine, First Faculty of Medicine, Charles University and General University Hospital in Prague, Prague, Czech Republic;

[2]2nd Department of Surgery – Department of Cardiovascular Surgery, First Faculty of Medicine, Charles University and General University Hospital in Prague, Prague, Czech Republic

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Key words: Extra-corporeal membrane oxygenation – Cardiac surgery – Thrombosis – Heart failure

Abstract: A massive left ventricular thrombosis represents a rare however, catastrophic complication of a central veno-arterial extra-corporeal membrane oxygenation. We report a case of such complication in a patient with severe left ventricular dysfunction after cardiac surgery. Its management and preventive measures are described and discussed.

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Mailing Address: Michal Pořízka, MD., PhD., E.D.I.C., Department of Anaesthesiology, Resuscitation and Intensive Medicine, First Faculty of Medicine, Charles University and General University Hospital in Prague, U Nemocnice 2, 128 08 Prague 2, Czech Republic; Mobile Phone: +420 606 945 580; e-mail: rizko@post.cz

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Introduction

Postcardiotomy heart failure represents a state with excessive morbidity and mortality in cardiac surgery. Veno-arterial extra-corporeal membrane oxygenation (VA-ECMO) is used as a mechanical heart support device when pharmacological inotropic support is ineffective (Fukuhara et al., 2016). Nevertheless, despite its beneficial effect on restoration of hemodynamic stability, there are many serious complications associated with its use. The most common are haemorrhage, severe infection and thromboembolism, all significantly increasing patient's morbidity and mortality (Makdisi and Wang, 2015).

Intracardiac thrombosis associated with VA-ECMO represents a rare, however catastrophic complication in terms of worsening morbidity and mortality related to the risk of systemic thrombus embolization (Williams and Bernstein, 2016). The insufficient unloading of left ventricle (LV) with blood stasis, use of artificial material of ECMO circuit and postoperative procoagulant state represent the most important risk factors for the intracardiac thrombus formation (Makdisi and Wang, 2015). There are only a few case reports describing this complication, thus the evidence for proper management and possible preventive measures is limited (Williams and Bernstein, 2016). Therefore, we present a case report of a patient on postoperative central VA-ECMO who developed a thrombosis of his left ventricular cavity.

Case report

A seventy-three-year-old male (height 170 cm, weight 68 kg) with known long standing history of a moderate aortic stenosis and worsening dyspnoea over the last three months was admitted to the regional hospital with a new onset of chest pain and an episode of pre-syncope. Shortly after the admission he developed a cardiogenic shock with respiratory failure due to pulmonary oedema. Patient had to be intubated and mechanically ventilated. A transoesophageal echocardiography (TEE) revealed a massive aortic regurgitation and severely dysfunctional LV – with ejection fraction of 20%. He was urgently transported to our cardio-vascular centre directly to operating theatre for further surgical management. At the admission patient was in profound circulatory shock state with the need of high vasopressor support (noradrenaline of 1 μg/kg/min). Intra-operatively, surgeon replaced severely calcified and degenerated bicuspid aortic valve with aortic bioprosthesis (Carpentier-Edwards Magna Aortic Heart Valve 25 mm, Edwards Lifesciences, USA). No vegetations or other signs of infective endocarditis were found. After the procedure a separation from cardio-pulmonary bypass was unsuccessful due to the extreme LV systolic dysfunction (EF of 10%) and patient was connected to VA-ECMO (centrifugal pump Maquet Rotaflow RF 32, Maquet Cardiopulmonary AG, Hirrlingen, Germany) with blood flow of 6 l/min. Arterial cannula was placed directly into the ascending aorta without the use of interposed vascular prosthesis and venous drainage cannula into the

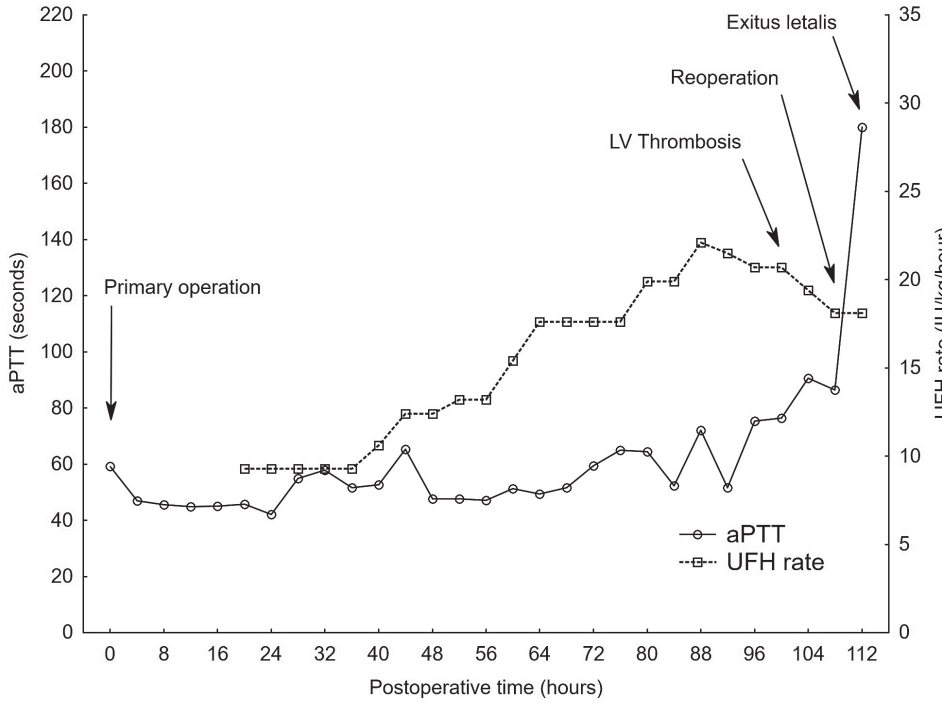

Figure 1 – Unfractionated heparin (UFH) infusion rates and corresponding activated partial thromboplastin times (aPTT) in the postoperative period.

right atrium. Primary sternotomy suture was performed with ECMO tubings placed into the jugular notch. Patient's hemodynamics stabilized on VA-ECMO and noradrenergic support decreased to 0.8 μg/kg/min. There was an excessive blood loss (650 ml in the first 12 hours postoperatively) due to cardio-pulmonary bypass induced coagulopathy (INR of 1.76), which was normalized by the administration of 6 units of fresh frozen plasma. Simultaneously anaemia was corrected by the transfusion of 3 units of packed red blood cells. Therefore, anticoagulation with unfractionated heparin (UFH) was started on the first postoperative day (POD), when the blood loss was minimal. Infusion rates of UFH and corresponding activated partial thromboplastin times (aPTT) during the postoperative course are depicted in the graph (Figure 1). Patient's clinical status on full VA-ECMO support further improved with continuous decrease of vasopressor requirements. Routine, every-day transthoracic echocardiography (TTE) controls showed a non-dilated, however akinetic left ventricle (LV) without any improvement in contractility even after the administration on inotropes (dobutamine of 10 μg/kg/min). Due to the development of tracheobronchitis on the second POD patient had to stay intubated and mechanically ventilated. On the fourth POD the TTE revealed an akinetic, non-dilated LV obliterated with a massive thrombus (Figure 2). Aiming

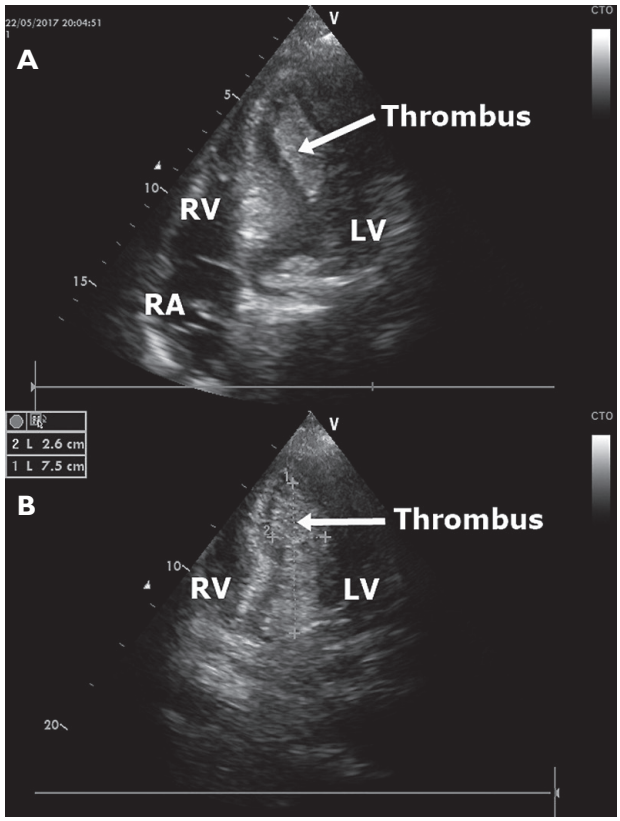

Figure 2 – Transthoracic echocardiographic images of the left ventricular thrombus. A – apical four-chamber view; B – apical five chamber view with the thrombus size measurements (LV – left ventricle, RA – right atrium, RV – right ventricle).

to evacuate the LV and left atrial (LA) thrombi the patient was reoperated. Intraoperatively other thrombi protruding into the LA from pulmonary veins were found and only partially extracted. The plan was to continue with left heart venting cannula inserted through left atrial appendage and to support contractility with the administration of inotropes. Nevertheless, immediate postoperative TEE was performed and revealed a recurrent massive LV and LA thrombosis. Simultaneously, there was an excessive postoperative bleeding (1000 ml/2 hours) due to severe cardio-pulmonary bypass induced coagulopathy (aPTT of 180 s, INR of 1.5). Patient's prognosis was evaluated as extremely poor and further therapy futile based of the fact of a massive recurrent heart thrombosis, severe coagulopathic bleeding, no LV recovery regardless of the inotropic support on the fifth POD and no eligibility for either long-term circulatory support or heart transplant due to the advanced age. A multi-disciplinary ECMO team decided to limit further therapy and to withdraw VA-ECMO support. Patient's family consented with this approach and patient was disconnected from VA-ECMO. The patient died shortly after the cessation of mechanical heart support.

Discussion

Thrombosis of LV cavity represents one of the most serious complications of VA-ECMO. Although it is a relatively common complication in patients with implanted left ventricular assist devices (Reilly et al., 2000), there are only few reports describing ventricular thrombosis in ECMO patients (Gaide-Chevronnay et al., 2012). There are several risk factors for the development of this complication, which were described for both central and peripheral venous cannulation.

The first major risk factor for intra-cardiac clotting is blood stasis in dilated, severely dysfunctional and non-ejecting heart not responding to an inotropic support. Such situation is especially unfavourable and problematic in peripheral VA-ECMO, in which a retrograde aortic blood flow is present, substantially increasing cardiac afterload for LV ejection (Gaide-Chevronnay et al., 2012). However, cases of LV thrombosis in central VA-ECMO with antegrade aortic blood flow have also been reported (Weis et al., 2009). On the other hand, several interventions exist that have the potential to prevent this complication. Firstly, the use of inotropes enhances contractility and augments cardiac ejections and may lead to partial cardiac decompression. This approach often fails in severe myocardial dysfunction, especially in the early phases after ECMO implantation, when unrecovered myocardium may be unresponsive to inotropes. Another way, how to achieve LV unloading, may be by either surgical or less effective percutaneous venting of LV (Meani et al., 2017). Additionally, an indirect LV unloading with intra-aortic balloon pump has been reported with variable success in VA-ECMO patients with partially preserved cardiac contractility (Doll et al., 2004). Finally, very promising possibility is adding Impella device that represents an alternative option to support antegrade flow from LV to aorta (Cheng et al., 2013), however it is not routinely available particularly due to its cost. Nevertheless, due to the insufficient data from clinical trials it is not clear which venting method is superior to another and its effect on mortality also remains unknown (Meani et al., 2017). A different approach compared to VA-ECMO is the use of left-ventricular assist device (LVAD) with the drainage cannula implanted directly into the LV, which has also been successfully used in patients with post-cardiotomy heart failure (Mebazaa et al., 2010). This method enables more efficient drainage of LV cavity compared to VA-ECMO, however fatal intra-cardiac thromboses have been also reported (Reilly et al., 2000). The disadvantages of LVAD in the immediate postoperative period include a need for biventricular support in patients with right ventricular dysfunction or a switch to ECMO circuit with oxygenator in a case of severe respiratory failure. It is also considerably more expensive compared to common VA-ECMO with centrifugal pump. In our institution we use CentriMag Ventricular Assist System (Levitronix, USA) as LVAD. However, in this specific case, the patient was not eligible for heart transplant or long-term LVAD due to the advanced age. Therefore, we decided for VA-ECMO only as a bridge to recovery in a patient who was in critical state with profound low cardiac output syndrome preoperatively,

thus with high risk of the development of multiple organ failure that is associated with extremely poor prognosis.

The second major risk factor represents a pro-coagulation state induced by systemic inflammatory response syndrome as a reaction to surgery with cardio-pulmonary bypass and ECMO artificial material (tubing, oxygenator). To prevent clotting in the ECMO circuit systemic anticoagulation is mandatory. The most widely used anticoagulation agent for provision of extra-corporeal life support is UFH. It is routinely given as a bolus infusion (50–100 units/kg of body weight) prior to initiation of ECMO and then infused continuously during ECMO support (The Extracorporeal Life Support Organization, 2014). However, no anticoagulation for a short period of time has been suggested in case of major bleeding especially in surgical patients (Marasco et al., 2008). Anticoagulation effect of UFH can be monitored using activated clotting time (ACT), aPTT or anti-Xa factor levels. The recommended levels of aPTT or ACT are 1.5–2.5 times of baseline values, however specific target values of these parameters for adequate systemic anticoagulation for ECMO patients are not precisely known (Bembea et al., 2013). The desired clinical effect of anticoagulation is also affected by concomitant coagulopathy, thrombocytopenia or hemodilution, which are common in these patients. Therefore, individualized anticoagulation thresholds should be used taking into account underlying coagulopathy, risk of bleeding in surgical patients and risk of ECMO circuit or cardiac thrombosis.

In our patient we started anticoagulation with UFH on the first POD (20 hours after surgery) because there was an excessive surgical bleeding and coagulopathy that had to be corrected in the immediate postoperative period. In the following two days we aimed for aPTT of 55–60 seconds (1.3–1.5 times of our laboratory baseline) due to the risk of surgical bleeding. Later on we targeted for aPTT of 60–80 seconds (1.5–2 times of baseline), which is our routine approach in non-surgical ECMO patients. From the perspective of our case it appears that we should have aimed for the higher aPTT level from the beginning. However, a re-thrombosis of LV developed despite very high level of aPTT (180 s) after reoperation. We did not suspect a heparin resistance as UFH rates were in the recommended range (The Extracorporeal Life Support Organization, 2014) for targeted aPTT levels (Figure 1). We also measured Antithrombin III (ATIII) levels every day, which were in the range of 50–60% throughout the postoperative course, and we did not supplement it. Due to the insufficient evidence, there is currently no recommended threshold for initiation of ATIII supplementation or desired ATIII levels targets for ECMO patients (The Extracorporeal Life Support Organization, 2014).

Another explanation for a postoperative pro-coagulation state is the development of type II heparin induced thrombocytopenia or presence anti-phospholipid syndrome. In the former case, aPTT is artificially prolonged and does not correspond to the true level of anticoagulation. Thus anti-Xa factor

levels would be a better parameter of anticoagulation monitoring. Nevertheless, we did not screen for pro-coagulation states due to the rapid clinical course after thrombosis occurred. Also no pro-coagulant disorders were known from patient's medical history and no medications with prothrombotic properties were administered to the patient pre- and postoperatively.

The other question is whether we should have vented LV earlier at the time of primary operation. LV venting via left atrium, which we used, is a well described surgical technique in decompressing LV (Weymann et al., 2014). However, it is not our routine approach, because it bears substantial surgical risk in terms of bleeding. We consider this technique only in very high risk patients with signs of significant blood stasis in LV cavity (severe LV dilatation and spontaneous echocardiographic contrast), which were not present at the time of primary operation. Unfortunately, at the end this approach was also ineffective in our patient.

In conclusion, massive LV thrombosis is rare, however devastating complication in patients on central VA-ECMO. Effective decompression of the heart, close monitoring of anticoagulation level and constant vigilance for the detection of pro-thrombotic states is of paramount importance to prevent its development and subsequent catastrophic consequences.

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Acquired Amegakaryocytic Thrombocytopenic Purpura Progressing into Aplastic Anemia

Jan Philipp Novotný, Birgit Köhler, Regina Max, Gerlinde Egerer
Department of Medicine V, University of Heidelberg, Heidelberg, Germany

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Abstract: Acquired amegakaryocytic thrombocytopenic purpura (AATP) is a rare hematological disorder characterized by severe thrombocytopenia and a complete or near-to complete absence of megakaryocytes in the bone marrow, while granulopoiesis, as well as erythropoiesis are usually preserved. Although autoimmune mechanisms are believed to be causative, the exact underlying pathogenesis is not known. To date, only few cases have been reported and management of this disease remains controversial with immunosuppression being the treatment modality of choice in the majority of patients. In this article, we report a case of newly acquired AATP without an associated autoimmune disease, refractory to corticoids, intravenous immunoglobulin (IVIG) and second-generation TPO (thrombopoietin) agonists, which have recently been approved for the treatment of thrombocytopenia. Finally, in accordance with other reports, disease progression into aplastic anemia has occurred.

Mailing Address: Jan Philipp Novotný, MD., Department of Medicine V, University Hospital Heidelberg, 69120 Heidelberg, Germany; Phone: +496 221 568 030; e-mail: JanPhilipp.Novotny@med.uni-heidelberg.de

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Introduction

Acquired amegakaryocytic thrombocytopenic purpura (AATP), also known as acquired pure megakaryocytic aplasia, is a rare hematological disorder reported in patients ranging from 2 to 89 years of age with predilection for males in the age group above 60 years (Figure 1). It can either occur isolated or be associated with systemic lupus erythematodus (SLE) (Cela et al., 2010; Ernestho-ghoud et al., 2015) and other autoimmune diseases (Hashimoto et al., 2016), as well as hematological malignancies such as non-Hodgkin lymphoma (Lugassy, 1996).

Most patients with this disease are initially diagnosed with immune thrombocytopenic purpura (ITP) and receive treatment with corticosteroids (either prednisone or dexamethasone) until a bone marrow aspirate or biopsy is performed, which reveals normal erythro- and granulopoiesis but near to complete absence of megakaryocytes (Hoffman et al., 1982), usually. Dysplastic changes consistent with myelodysplastic syndromes are not seen initially, but may appear in the clinical course of the disease with progression to myelodysplastic syndrome (Erkurt et al., 2005). Furthermore, progression to aplastic anemia (Meytes et al., 1984; King et al., 1997) and association with hematologic malignancies (Geissler et al., 1987), as well as presence of concurrent anemia has been reported (Niparuck et al., 2008).

The exact underlying pathogenic mechanisms remain unknown to some extent, but successful treatment with immunosuppressive agents points towards an autoimmune-mediated process. The variable outcome with different treatments, however, is indicative of not only a singular mechanism of pathogenesis. Indeed, inhibition at the level of the thrombopoietin receptor (Hoffman et al., 1989) due to a humoral agent, antibodies against thrombopoietin itself (Katsumata et al., 2003) or a T-cell mediated process (Gewirtz et al., 1986; Doubek et al., 2006) have been described.

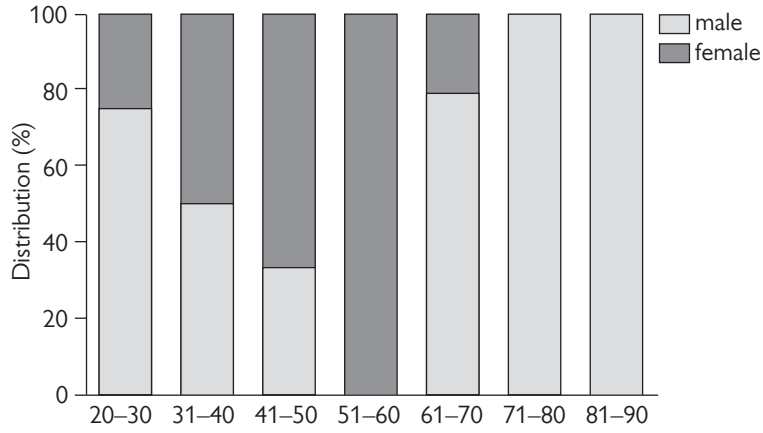

Figure 1 – Graphical representation of age and gender distribution of 30 patients with diagnosed AATP.

In contrast to that, congenital amegakaryocytic thrombocytopenia (CAMT) is characterized by a mutation in the *MPL* gene coding for the TPO (thrombopoietin) receptor and is inherited in an autosomal recessive manner. Depending on the type of mutation, CAMT can be further classified into type-I or type-II according to the type of underlying mutation predicted to result in either complete loss of function or some retained function of the TPO receptor (nonsense and missense mutation, respectively) (King et al., 2005). However, CAMT without mutation in the *MPL* gene has been described in association with other diseases (e.g. Hoyeraal-Hreidarsson syndrome) and is referred to as type-III (King et al., 2004).

To date, allogeneic hematopoietic stem cell transplantation remains the only therapeutic option, whilst gene therapy or TPO agonists binding to partially functioning TPO receptors might provide benefit in the future.

Finally, AATP and CAMT have to be, amongst other things, distinguished from ITP, which is characterized by either an increased degradation of thrombocytes due to autoantibodies (Cines et al., 2009), inhibition of thrombocytopoiesis by autoantibodies interfering with megakaryopoiesis (Chang et al., 2003; McMillan et al., 2004), T-cell mediated toxicity towards thrombocytes (Olsson et al., 2003) or relatively insufficient thrombopoietin concentration (Emmons et al., 1996).

No primary therapy for AATP has been established to date. Immunosuppression, however, remains the mainstay of therapy. Treatments shown to be successful include the use of corticoids (Sakurai et al., 1984), intravenous immunglobulins (IVIG) (Leach et al., 1999), cyclosporine (Omri et al., 2010), anti-thymocyte globulin (ATG) (Niparuck et al., 2008), as well as allogeneic stem cell transplantation (Lonial et al., 1999) and anti-CD20 antibodies (Deeren and Dorpe, 2010; Mirzania et al., 2014). While these and other therapeutic agents have shown a wide range of response rates, reports emerged reporting success with thrombopoietin (TPO) receptor agonists recently; a treatment option that has already been theorized earlier (Lown et al., 2010). Published case reports include the use of Elthrombopaq in a patient with AATP and associated systemic lupus erythematosus (SLE) (Cela et al., 2010), as well as Romiplostim in a patient without underlying autoimmune disease (Shigekiyo et al., 2015).

Material and Methods

The literature review was performed with searches on google scholar using the keywords "acquired amegakryocytic", "acquired pure megakaryocytic aplasia". Statistical analysis was performed on 30 patients (including the patient from this report) published in 19 articles. For this, only publications and case reviews reporting adult patients without significant comorbidities and autoimmune disease have been included. Gender, age and treatment modality have been analysed using Excel. Patients receiving simultaneous treatment with more than one agent have been excluded from response rate analysis.

Case report

A 61-year-old Caucasian male with a history of recent epistaxis, easy bruising and petechiae, which developed suddenly two weeks before admission. The patient history includes a perforated sigma diverticulitis 9 years ago, a total endoprothesis of the knee 2006, as well as a peripheral arterial occlusive disease stage IIa, adiposity with a BMI (body mass index) of 35 and a nicotine abuse of 45 pack years. No allergies or intolerances are known. On admission in a peripheral hospital due to severe epistaxis, blood workup showed marked thrombocytopenia of 0/nl and makrocytic, hyperchromic anemia (10.2 g/dl; Table 1), which worsened progressively with values reaching 7.4 g/dl five days later, probably due to several episodes of intermittent epistaxis. EDTA (ethylenediaminetetraacetic acid) induced pseudothrombocytopenia was ruled out.

Serologically there was no evidence of a hepatitis B, C or HIV infection. Vitamin B_{12} concentration was normal, folic acid was mildly reduced at 4 ng/l and substitution was started until concentration above the upper normal limit was detected. Autoimmune diagnostics showed normal lupus anticoagulans concentration as well as a normal lupus sensitive – apTT. No thrombocyte antibodies were detected, coombs test was negative. An ultrasound of the abdomen showed no splenomegaly and lactate dehydrogenase (LDH) was within normal limits.

Suspecting a case of immune thrombocytopenia the patient initially received a prednisone burst with 250 mg/day and, due to refractory thrombocytopenia, subsequently 80 g intravenous immunoglobulins, which did not lead to a rise in

Table 1 – Initial and subsequent blood counts

	Initially	Approx. 1 month later	Approx. 14 months later	Unit
Hemoglobin	10.2	8.4	8.9*	g/dl
Hematokrit	30.0	0.24	0.25	l/l
Erythrocytes	3.1	2.5	3.1	/pl
MCH	33.0	34.0	29.0	pg
MCHC	34.0	35.0	36.0	g/dl
MCV	98.0	97.0	81.0	fl
Leukocytes	6.05	4.46	0.57	/nl
Thrombocytes	0.0	8.0	22.0*	/nl
Neutrophils	64.0	59.2		%
Lymphocytes	22.0	29.4		%
Monocytes	9.3	7.5		%
Eosinophils	4.5	1.3		%
Basophils	0.2	0.0		%

*transfusions received; MCH – mean corpuscular hemoglobin; MCHC – mean corpuscular hemoglobin concentration; MCV – mean corpuscular volume

thrombocyte counts. Due to symptomatic anemia the patient received several erythrocyte transfusions. He was then referred to our clinics for further diagnostics and treatment.

Upon admission we decided to try another cycle of corticoids (Dexamethasone 40 mg/day, 4 days) and intravenous immunoglobulins at a concentration of 1 g/kg body weight (BW) (110 g) to confirm the refractoriness to these immunosuppressive agents. We also performed bone marrow cytology, flow cytometry of bone marrow blood and a bone marrow biopsy with subsequent histological examination, as well as chromosomal analysis. The cytology showed a near to complete absence of megakaryocytes with preserved granulo- and erythropoiesis. Slight dysplastic changes such as minimal misshaped nuclei and pseudo-pelger cells could be seen upon cytological examination. Ring sideroblasts were not observed.

Repeated chromosomal analysis showed normal male karyotype XY, 46. Flow-cytometry revealed no lead towards myelodysplasia and histological examination of the bone marrow biopsy confirmed the presence of normal maturation of the white and red lineage with nearly total absence of megakaryocytes as well as minimal increase in interstitial reticular fibers. There was no sign of an intramedullary increase in CD34+ cells and no infiltration by lymphoid cells. Erythropoietin concentration was well above normal limits.

During the in-hospital stay the patient developed fever with temperatures around 39 °C, which was initially treated with Tazobactam/Piperacillin i.v. and then escalated to Meropenem i.v., and empirical Caspofungin i.v., as well as Clarithromycin p.o. after several days in a stepwise manner prior to further diagnostics due to suspicion of atypical pneumonia. Blood cultures showed no bacterial growth. In order to localize the focus of the suspected infection a CT (computed tomography) scan of the chest was performed which showed no pneumonia, but incidentally revealed an 2×1.4 cm adrenal adenoma with partially negative Hounsfield units. The fever resolved during the course of treatment and

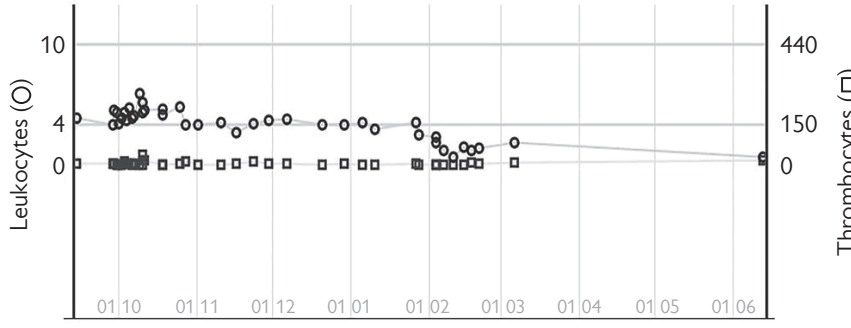

Figure 2 – Thrombocyte and leukocyte counts during therapy.

C-reactive protein concentration decreased accordingly so that antibiotic and antimycotic treatment was discontinued.

In summary of the results we then diagnosed the patient with acquired amegakaryocytic thrombocytopenic purpura. Due to the risks associated with immunosuppressive agents such as Ciclosporine, or Cyclophosphamide we decided to initiate a therapy with 50 mg/day Elthrombopaq, a TPO receptor agonist available for treatment of immune thrombocytopenia (ITP), which was successfully used in a patient with AATP and systemic lupus erythematosus (SLE) (Cela et al., 2010). After several days of treatment with Elthrombopaq we initially observed a rise in thrombocyte count and a decreased tendency to bleed. Thrombocyte counts, however, fell again after several weeks and the dose subsequently increased by 25 to 75 mg/day. Laboratory follow-ups showed no improvement of the thrombocyte count after several weeks, necessitating another hospitalization and change of treatment to a combination therapy of Romiplostim and Ciclosporine, which again did not result in thrombocyte counts > 50/nl (Figure 2). Repeated bone marrow histology then showed fatty degeneration, absent megakaryopoiesis and little granulo/erythropoiesis. In accordance to that, peripheral blood analysis revealed progressive leukopenia, as well as anemia, necessitating further erythrocyte transfusions. Finally, the diagnosis of aplastic anemia was made. Further workup showed inconspicuous JAK2-exon12 sequencing and cytomegalovirus infection was ruled out. Sequencing of *ASXL1*, *CBL*, *DNMT3A*, *EZH2*, *RUNX*, *SF3B1*, *SRSF2*, *TET2*, *TP53*, *U2AF1*, *ZRSR2* did not reveal any mutations other than SNPs in *TP53* and *EZH2*. *BCOR*, *BCORL1*, *DNMT3A* sequencing was uneventful.

With regards to the progressive leukopenia, as well as aplastic anemia further therapy with an anti-CD20 antibody was deemed unsuitable at that time point and work-up for allogeneic stem cell transplantation has been started.

Discussion

The distribution of AATP among gender is equal, but differs according to age. While most affected females are in the 40–60 age, more men are affected at both ends of age distribution (Figure 1) with a peak in the 60ties.

Analysis of published reports reveals a success rate of <20% using either corticoids or IVIG alone. Treatment with Ciclosporine yielded a response rate of about 50% compared to 80% with anti-thymocyte globulin (ATG). Two cases were successfully treated with anti-CD20 antibodies, while there is only one case report of successful allogeneic stem cell transplantation in a patient refractory to multiple immunosuppressive agents and one report on successful use of Mycophenolate Mofetil (Bulchandani et al., 2007) (Table 2). In scarce reports, anti-CD20 antibodies and TPO receptor agonists showed 100% response (N=2 each).

With less than a 20% response rate, corticoids and IVIG are insufficient as first line therapy in AATP. Treatment with ATG seems promising, but carries the risk of allo-immunization and requires in-hospital stay. Ciclosporine on the other

Table 2 – Percent response to specific therapy

Treatment	Response rate (%)	N
Corticoids	9	22
Intravenous immunoglobulin	14	7
Ciclosporine	50	6
Anti-thymocyte globulin	80	5

hand greatly increases the rate of infections, as well as the risk of liver damage necessitating close monitoring of liver enzymes and kidney function. Whether anti-CD20 therapy represents a better therapeutic option remains to be assessed. Due to the serious side effects accompanied with an allogeneic stem cell transplantation, we feel that this treatment option should be reserved for those in certain age, refractory to more than one therapeutic option other than corticoids or IVIG and absence of significant comorbidities. TPO receptor agonists seem to be a reasonable first line therapy due to their mechanism of action even despite our negative result.

Our case report shows the importance of complete diagnostics in patients presenting with apparent immune thrombocytopenia and their regular follow-ups. It unrolls the lack of an optimal treatment algorithm for AATP and depicts the importance of case reports for this entity in order to assess treatment success with different therapeutic regimens, since prospective randomized clinical studies are difficult to perform due to the rarity of the disease.

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Annual Contents

No. 1

No. 2–3

No. 4

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