

OncoImmunology

ISSN: (Print) 2162-402X (Online) Journal homepage: http://www.tandfonline.com/loi/koni20

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To cite this article: Elisabetta Ferrero, Angelo Corti, Julien Haroche, Daniela Belloni, Barbara Colombo, Alvise Berti, Giulio Cavalli, Corrado Campochiaro, Antonello Villa, Fleur Cohen-Aubart, Zahir Amoura, Claudio Doglioni, Lorenzo Dagna & Marina Ferrarini (2016) Plasma Chromogranin A as a marker of cardiovascular involvement in Erdheim–Chester disease, OncoImmunology, 5:7, e1181244, DOI: 10.1080/2162402X.2016.1181244

To link to this article: http://dx.doi.org/10.1080/2162402X.2016.1181244

Accepted author version posted online: 05 May 2016.
Published online: 05 May 2016.

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ONCOIMMUNOLOGY
2016, VOL. 5, NO. 7, e1181244 (7 pages)
http://dx.doi.org/10.1080/2162402X.2016.1181244

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ORIGINAL RESEARCH

Plasma Chromogranin A as a marker of cardiovascular involvement in Erdheim–Chester disease

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ABSTRACT

Erdheim–Chester disease (ECD) is a rare non-Langerhans cell histiocytosis (LCH) characterized by tissue infiltration with $CD68^+$ foamy histiocytes. TNF-related chronic inflammation and mutations in the MAP kinase signaling pathway in histiocytes are recognized as the two major pathogenic events. Among pleomorphic clinical manifestations, cardiovascular involvement is frequent and prognostically relevant. Evaluation of ECD clinical course and response to treatment is, however, still challenging. Taking advantage of the two largest cohorts of ECD patients worldwide, we investigated the relevance and the potential of circulating Chromogranin A (CgA), a pro-hormone involved in cardiovascular homeostasis and inflammation, as a biomarker of response to therapy in ECD. Consistent with other TNF-related inflammatory diseases, we found that not only TNF-α and soluble TNF-Receptors (sTNF-Rs), but also CgA plasma levels were significantly increased in ECD patients compared to controls. CgA, but not sTNF-Rs, discriminated cardiovascular involvement in ECD patients and correlated with pro-Brain Natriuretic Peptide (pro-BNP). In a single case, where a cardiac biopsy was available, CgA was found expressed by cardiomyocytes but not by infiltrating histiocytes. In four ECD patients, where serial determination of these parameters was obtained, the kinetics of sTNF-Rs and CgA paralleled response to therapy with anti-cytokine inhibitors; specifically, sTNF-Rs overlapped TNF-associated inflammation, while CgA, together with pro-BNP, closely mirrored response of cardiac disease. Our data indicate that both sTNF-Rs and CgA are linked to ECD pathophysiology. Moreover, CgA, in concert with pro-BNP, can be further exploited to fulfill the unmet clinical need of non-invasive reliable biomarkers of cardiac disease in these patients.

Abbreviations: CgA, Chromogranin A; CHF, chronic heart failure; ECD, Erdheim–Chester disease; LCH, Langerhans cell histiocytosis; pro-BNP, pro-Brain Natriuretic Peptide; sTNF-R, soluble TNF-Receptors; TNF-α, Tumor Necrosis Factor-α

ARTICLE HISTORY
Received 13 January 2016
Revised 14 April 2016
Accepted 15 April 2016

KEYWORDS
Chromogranin A; chronic inflammation; Erdheim–Chester disease; histiocytes; soluble TNF-Receptors; TNF-α

Introduction

Erdheim–Chester disease (ECD) is a rare form of non-Langerhans cell histiocytosis (LCH), with about 550 cases reported since its first description in 1930.[1] Histologically, the disease is characterized by xanthomatous or xantho-granulomatous infiltration of tissues by foamy $CD68^+$ $CD1a^-$ non-Langerhans histiocytes or lipid-laden macrophages, surrounded by fibrosis.[2-6] Since virtually any organ can be affected, ECD exhibits protean clinical manifestations, including the nearly pathognomonic osteosclerosis especially of the long bones and extra-skeletal manifestations (exophthalmos, xanthelasma, interstitial lung disease, obstructive renal impairment, diabetes insipidus, and cardiovascular and central nervous system (CNS) involvement).[2-6] The prognosis is generally severe, particularly when the cardiovascular and central nervous system are involved.[5] Given the lack of reliable biomarkers, evaluation of clinical course of ECD and response to treatment is still challenging.[5]

We and others have identified two major and possibly interconnected pathogenic events, i.e., a full-blown chronic inflammation and a mutation in the Ras–Raf–Mek–Erk signaling pathway (in particular the $BRAF^{V600E}$ mutation) in histiocytes, which is detectable in the majority of ECD patients.[7-11] Oncogene-induced senescence has been proposed as a possible link between those pathogenic events,[12] which have been therapeutically targeted with cytokine inhibitors and more recently with the $BRAF^{V600E}$ inhibitor vemurafenib.[13-16]

Chronic local and systemic inflammation is a hallmark of the disease,[7,8] with TNF-α playing a key role, as indicated by increased plasma levels of TNF-related cytokines and chemokines, and also of soluble TNF-Receptor I (sTNF-RI) and sTNF-RII, in ECD patients.[14] sTNF-Rs derive from proteolytic cleavage of membrane-associated TNF-Rs, which is promoted by TNF-α itself, and can therefore be considered *bona fide* surrogates of TNF-activity.[17] Recently, serum TNF-α and/or

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sTNF-Rs levels have been found to correlate with increased circulating Chromogranin A (CgA) in inflammatory diseases and cancer.[18-21]

CgA is 49-kDa acidic polypeptide stored in the secretory granules of chromaffin cells and released in the extracellular environment by exocytosis. This protein can undergo intracellular and extracellular proteolytic processing, originating biologically active fragments which are implicated in several functions, including vascular homeostasis, angiogenesis, and tissue repair.[22-25] CgA levels are increased in patients with neuroendocrine tumors, or with hearth or renal failure, rheumatoid arthritis (RA) or other inflammatory diseases.[18-22] With regard to the cardiovascular system, serum CgA levels correlate with severity of cardiac dysfunction and are a predictive factor for mortality in patients with chronic heart failure (CHF) and acute coronary syndrome.[21,26-28] More recently, immunohistochemical analyses identified cardiomiocytes as an important source of CgA in patients with CHF.[29] In these cells, CgA co-localized with the brain natriuretic peptide (BNP) inside cytoplasmic granules in patients but not in normal controls.[29]

The relationship between CgA and TNF-α/sTNF-Rs in ECD, and also its association with cardiovascular involvement, a prominent clinical manifestation of the disease,[30,31] are presently unknown. Taking advantage of the two largest cohorts of ECD patients worldwide, we aimed to assess the expression and kinetics of circulating sTNF-Rs and CgA in ECD patients, in order to explore their potential to fulfill the unmet clinical need of non-invasive reliable biomarkers of disease activity and response to therapy.

Results

Circulating CgA is increased in ECD patients and identifies cardiovascular involvement

We have previously reported the systemic increase in TNF-α, TNF-related cyto-chemokines and sTNF-Rs in 10 ECD patients.[14] We here confirm and extend this observation to a larger cohort of patients, including 17 patients from Ospedale San Raffaele (OSR) and 20 from Pitié-Salpêtrière Hospital. Demographic and clinical characteristics of ECD patients recruited to the study are summarized in Table 1. Also in this cohort of patients sTNF-RI and -RII are significantly increased compared to controls (Table 2). Circulating CgA was also significantly increased in ECD patients (Table 2), and correlated with sTNF-RI only ($r = 0.433$, $p \leq 0.05$); sTNF-RI and sTNF-RII instead strongly correlated with each other ($r = 0.770$, $p \leq 0.001$) (Fig. 1A).

Given the reported increase in circulating CgA in patients affected by CHF,[20] we aimed to determine whether CgA levels

Table 1. Demographic and clinical characteristics of ECD patients.

	Age	Sex	Involvement	Ongoing therapy
1	69	F	Exophthalmos, diabetes insipidus, bone, pleuro-pericardial effusion	None
2	49	M	Bone, retroperitoneal fibrosis, lung	None
3	46	M	Retroperitoneal fibrosis, diabetes insipidus, exophthalmos, bone	PEG-IFNα
4	52	M	End-stage renal disease, heart (chronic heart failure, pericardial effusion)	None
5	79	F	Heart (CHF), mediastinal localization, bone	Corticosteroids
6	65	F	Bone, lung, heart, xanthelasma, CNS	IFNα
7	46	M	Retroperitoneal fibrosis, renal failure, aortic aneurism, lung, bone	Corticosteroids + IFNα
8	61	M	Heart (CHF), bone, lung, diabetes insipidus, hypogonadotropic hypogonadism, retroperitoneal fibrosis	Methotrexate
9	56	M	Heart (atrial mass and pericardial effusion), retroperitoneal fibrosis, bone	Corticosteroids
10	71	M	Heart (pericardial effusion), bone, retroperitoneal fibrosis	none
11	54	M	CNS, diabetes insipidus	IFNα
12	55	M	Renal failure, retroperitoneal fibrosis, bone, xanthelasma, CNS and heart (atrial mass and pericardial effusion)	IFNα
13	27	M	Diabetes insipidus, CNS, renal failure, bone, pulmonary fibrosis	Corticosteroids
14	26	M	Retroperitoneal fibrosis, CNS, retro-orbital, pericardial effusion, bone	Corticosteroids + IFNα
15	45	M	Diabetes insipidus, pulmonary fibrosis, optic nerves, retroperitoneal fibrosis, bone	Corticosteroids
16	58	F	Retroperitoneal fibrosis, xanthelasma, bone	IFNα
17	52	M	Pan-hypopituitarism, diabetes insipidus, pulmonary infiltration, bone	Corticosteroids
18	69	M	CNS, bone	PEG-IFNα
19	25	F	Skin, exophthalmos, bone	IFNα
20	33	M	CNS, exophthalmos, retroperitoneal fibrosis, bone	Cladribine
21	64	F	Xanthelasma, heart (atrial mass and pericardial effusion), retroperitoneal fibrosis, bone	IFNα
22	59	M	Xanthelasma, bone, heart (pericardial effusion), diabetes insipidus, CNS, retroperitoneal fibrosis	IFNα
23	58	F	Bone, exophthalmos, CNS, retroperitoneal fibrosis	None
24	45	F	Bone, xanthelasma, retroperitoneal fibrosis, diabetes insipidus	PEG-IFNα
25	67	F	Bone, xanthelasma, heart (atrial mass and pericardial effusion), lung, retroperitoneal fibrosis	Corticosteroids
26	73	M	Bone, retroperitoneal fibrosis, xanthelasma, CNS	Corticosteroids + IFNα
27	65	F	Bone, xanthelasma	PEG-IFNα
28	60	M	Bone, diabetes insipidus, heart (pericardial effusion), retroperitoneal fibrosis	PEG-IFNα
29	66	M	Bone, lung, retroperitoneal fibrosis	IFNα
30	75	M	Bone, CNS, diabetes insipidus, retroperitoneal fibrosis	IFNα
31	33	M	Bone, heart (atrial mass), retroperitoneal fibrosis, CNS, xanthelasma	Corticosteroids
32	68	F	Bone, retroperitoneal fibrosis, lung, xanthelasma, exophthalmos	Corticosteroids
33	55	M	Retroperitoneal fibrosis, bone, exophthalmos	None
34	56	M	Heart (atrial mass and pericardial effusion), retroperitoneal fibrosis, aortic aneurism, bone	PEG-IFNα
35	52	M	CNS, diabetes insipidus, bone, exophthalmos	None
36	63	M	Heart (CHF), coated aorta, bone	None
37	52	M	CNS, diabetes insipidus, bone, retroperitoneal fibrosis	PEG-IFNα

Abbreviations: CNS, central nervous system; CHF, chronic heart failure; IFN, interferon.

Table 2. Soluble TNF-Rs and Chromogranin A in ECD patients.

	sTNF-RI (ng/mL)	sTNFR-II (ng/mL)	TNF-[§] (pg/mL)	CgA (ng/mL)
HD (n = 20)	1.21 ± 0.09	1.83 ± 0.10	0.89 ± 0.56	35.4 ± 1.7
ECD (n = 37)	3.88 ± 0.41***	4.86 ± 0.35***	11.53 ± 3.41*	153.08 ± 23.7**

Results are mean ± SEM of soluble TNF Receptor (sTNF-R) I and II, TNF-, and Chromogranin A (CgA) concentrations in plasma from Healthy Donors (HD) and ECD patients, as determined by ELISA

[§]TNF- concentration was determined in 17 ECD patients and 7 HD. Data obtained from ECD patients and controls were compared by the Mann– Whitney U test

*statistically significant at $p < 0.05$.

**statistically significant at $p < 0.01$.

***statistically significant at $p < 0.001$.

in ECD correlate with cardiac disease. Since echocardiography is not sensitive enough to exclude cardiac involvement,[5] we analyzed only 17 patients who had cardiac MRI performed at the time of blood sampling. Baseline clinical characteristics of these patients (eight with cardiac involvement and nine without) are summarized in Table 3. CgA concentration in patients with cardiac involvement was significantly higher (217.66 ± 38.29 ng/mL) compared to both HD (35.44 ± 1.72 ng/mL; $p \leq 0.001$) and nine ECD patients without cardiac involvement (84.36 ± 21.48 ng/mL; $p \leq 0.001$) (Fig. 1B, Table 4). Neither sTNF-R could discriminate between the two groups of patients (Fig. 1B, Table 4): sTNF-RI and sTNF-RII concentrations were 4.41 ± 0.99 ng/mL and 4.22 ± 0.84 ng/mL in ECD patients with versus 4.55 ± 1.04 ng/mL and 5.30 ± 1.05 ng/mL in patients without cardiac involvement.

Since circulating CgA significantly correlates with BNP in patients with cardiomyopathy,[29] we determined Pro-BNP levels in 14 ECD patients, including 8 with and 6 without cardiac involvement. Pro-BNP levels were higher in the former group (median value of 613.5 pg/mL, range 42–5196 pg/mL vs. 124 pg/mL, range 17–366 pg/mL). Moreover, pro-BNP levels significantly correlated with circulating CgA (r = 0.618, $p \leq 0$

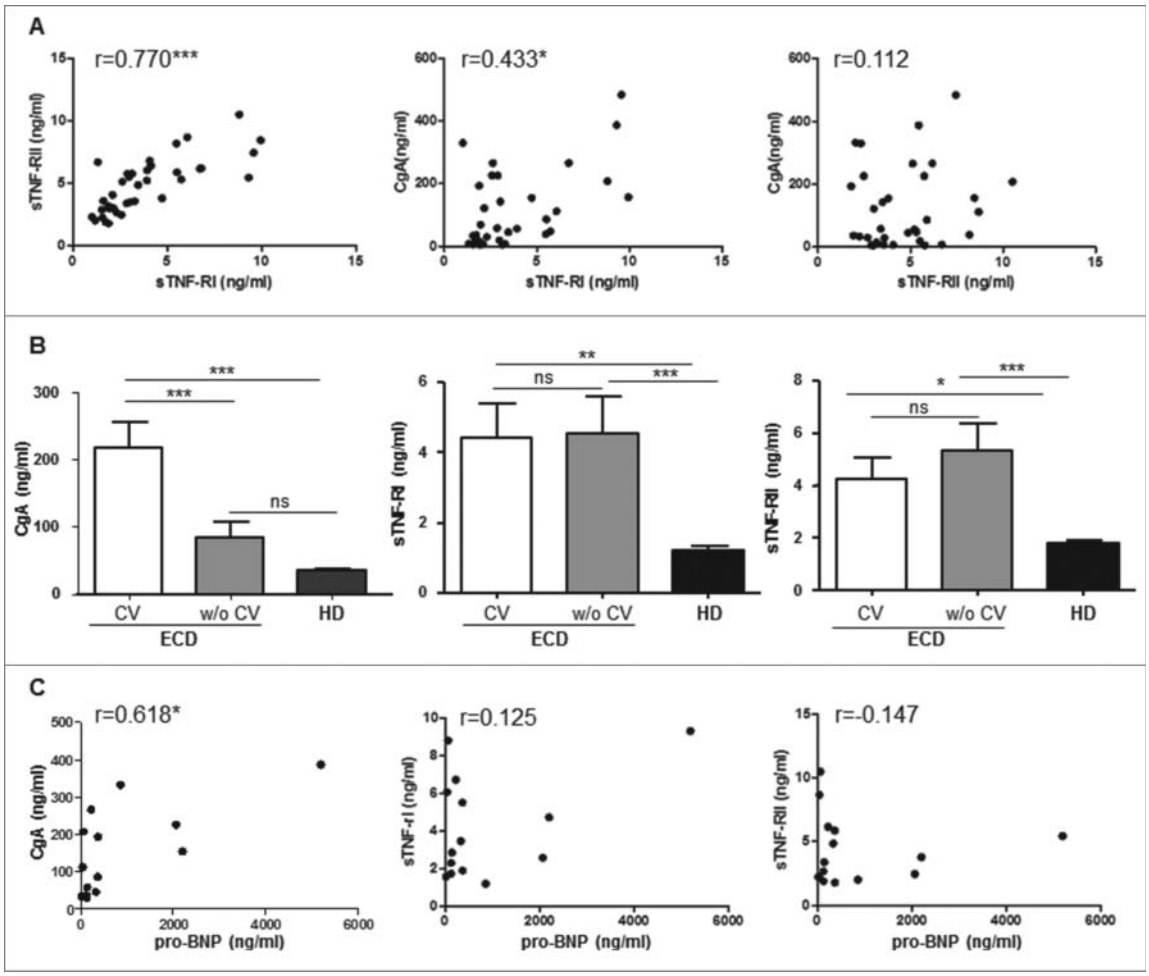


Figure 1. Circulating CgA identifies cardiovascular involvement in ECD patients. (A) Correlation between sTNF-RI and sTNF-RII (left) and between sTNF-RI and CgA (middle) and sTNF-RII and CgA (right) was evaluated by Spearman correlation analysis in 37 ECD patients. (B) circulating CgA, sTNF-RI, and sTNF-RII levels were determined in ECD patients with (n = 8, white columns) or without (w/o) (n = 9, gray columns) cardiovascular involvement (CV) and represented as mean ±SEM . Statistical significance was assessed by ANOVA. Black columns represent the mean of healthy controls (HD) (n = 20). (C) correlation between circulating CgA, sTNF-RI, or sTNF-RII with pro-BNP values was performed by Spearman correlation analysis in 14 patients. *$p < 0.05$; **$p < 0.005$; ***$p < 0.001$.

Table 3. Characteristics of ECD patients with or without cardiovascular involvement.

	ECD with CV involvement (n = 8)	ECD without CV involvement (n = 9)
Median systolic blood pressure (mmHg)	135	125
Median diastolic blood pressure (mmHg)	75	80
Arterial hypertension (% of patients)	88%	56%
Median LV ejection fraction (%)	67	65
Median LV end diastolic volume (mL)	93	116
Diastolic dysfunction (% of patients)	75%	33%
Systolic disfunction (% of patients)	13%	11%
Median pericardial effusion thickness (mm)	16.4	N.A.

Abbreviations: LV, left ventricular; CV, cardiovascular; N.A., not applicable.

.05) (Fig. 1C). Neither sTNF-RI nor sTNF-RII correlated with pro-BNP (Fig. 1C).

CgA is expressed in cardiomyocytes from an ECD patient

Cardiomyocytes from patients with dilated cardiomyopathy and hypertrophic cardiomyopathy have been identified as a source of CgA.[29] We therefore investigated intra-lesional CgA expression on a cardiac biopsy obtained for diagnostic purposes from an ECD patient who presented with a cardiac mass, involving the right atrium and the right intra-ventricular groove.[14] Infiltration by foamy histiocytes (Figs. 2A–C), which stained positive for CD68 (Fig. 2B), was evident, leading to ECD diagnosis.[5] Notably, intra-lesional cardiomyocytes expressed the reported diffuse granular cytoplasmic immunoreactivity for the anti-CgA antibody 5A8 (Figs. 2C–D),[29] at variance with infiltrating histiocytes.

sTNF-Rs and CgA levels parallel response to therapy

To investigate the potential of sTNF-Rs and CgA as biomarkers of response to therapy in ECD patients, we serially determined these parameters in four patients with cardiovascular involvement and divergent response to therapy (infliximab for Patients 8 and 10; tocilizumab for Patients 9 and 11). In particular, Patients 9 and 10 experienced substantial amelioration of their cardiac disease upon treatment; Patient 8 showed an initial response of his cardiac disease, which later progressively worsened, and Patient 11 presented neurological progression under treatment, while showing improvement of cardiac involvement.

As shown in Fig. 3, the time-course of all soluble factors paralleled response to therapy. The kinetics of circulating sTNF-Rs and CgA, however, did not completely overlap. In fact, in patients with prominent cardiovascular involvement (Patient 8 and particularly Patients 9 and 10), CgA and pro-BNP levels marked the clinical course of cardiac disease. Conversely, in Patient 11, where response to treatment discriminated neurological from cardiac involvement, sTNF-Rs levels steadily increased at variance with the decrease of CgA and pro-BNP levels.

In all patients, TNF-α plasma levels paralleled circulating sTNF-Rs (Fig. 3).

Discussion

ECD is a rare form of non-LCH, characterized by mutations in the Ras–Raf–Mek–Erk signaling pathway in histiocytes, associated with local and systemic chronic inflammation. We here confirm the key pathogenic role of TNF-α in a large cohort of 37 ECD patients, showing an increase in both plasma TNF-α and sTNF-Rs. TNF-α is produced by intra-lesional ECD histiocytes in the context of a complex cytokine-chemokine storm,[7,32] and in turn can modulate the release of down-stream pro-inflammatory molecules and also of sTNF-Rs.[14] Excessive TNF-α exerts its detrimental effects particularly on vasculature;[17] consistently, we have recently reported that TNF-α released in pericardial fluid from ECD patients promotes vascular leakage *in vitro*.[33] sTNF-Rs counteract TNF-driven inflammation, possibly in concert with CgA, which is released from neuroendocrine cells in response to TNF-α and also inhibits TNF-induced endothelial activation.[22-25]

Another major source of CgA is represented by stretched cardiomyocytes from patients with dilated and hypertrophic cardiomyopathy, where it co-localizes with BNP.[29] The emerging pathophysiological contribution of CgA to cardiovascular homeostasis is further suggested by its inhibitory effect on myocardial contractility and relaxation found in experimental models.[34] Of note, we here show for the first time that CgA is expressed also by cardiomyocytes inside ECD intracardiac masses. This finding, together with the correlation of CgA levels with cardiovascular involvement and with pro-BNP, suggests that, also in ECD, CgA is mainly produced and released by diseased myocardium and may be implicated in cardiovascular homeostasis and tissue remodeling.[29]

Finally, we investigated the potential of sTNF-Rs and CgA as biomarkers for ECD. Assessment of disease activity and response to therapy presently relies on clinical examination and radiologic investigation.[5] Recently, a droplet-digital assay for quantitative determination of $BRAF^{V600E}$ mutation in plasma and urine cell-free DNA was proposed as a sensitive biomarker of response to therapy in both ECD and LCH

Table 4. Soluble TNF-Rs and Chromogranin A in ECD patients with/without cardiovascular involvement.

	sTNF-RI (ng/mL)	sTNF-RII (ng/mL)	CgA (ng/mL)
ECD w CV (n = 8)	4.41 ± 0 .99**	4.22 ± 0 .84*	217.66 ± 38 .29***
ECD w/o CV (n = 9)	4.55 ± 1 .04***	5.30 ± 1 .05***	84.36^{ns} ± 21 .48
HD (n = 20)	1.21 ± 0 .09	1.83 ± 0 .10	35.44 ± 1 .72

Results are mean ± SEM of soluble TNF Receptor (sTNF-R) I and II, and Chromogranin A (CgA) concentrations in plasma from ECD patients with or without cardiovascular involvement (CV) and from Healthy Donors (HD), as determined by ELISA. Statistical significance between ECD patients and controls was assessed by ANOVA.
*statistically significant at $p < 0$ .05.
**statistically significant at $p < 0$ .01.
***statistically significant at $p < 0$ .001.
n.s. = not significant.

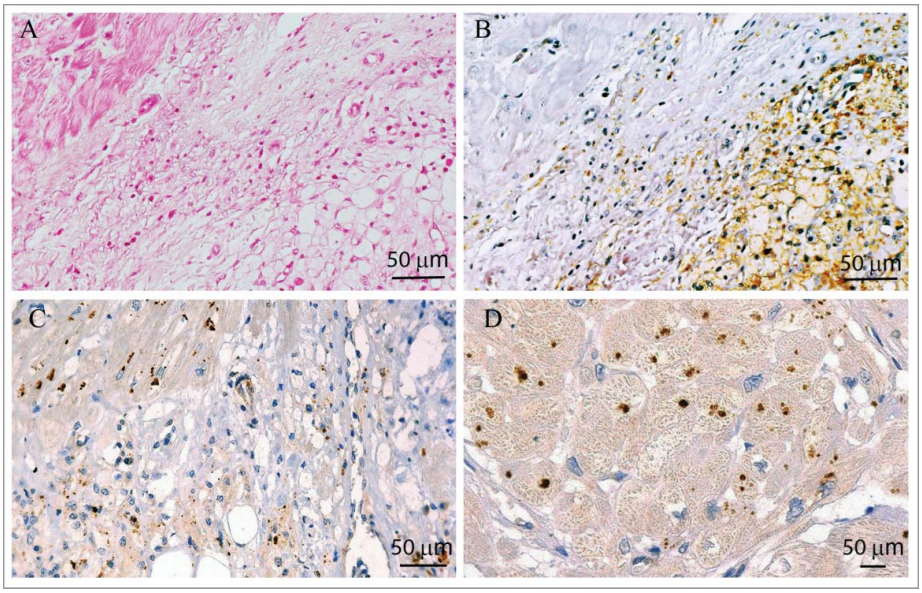


Figure 2. ECD cardiomyocytes express CgA. Immunohistochemistry on ECD myocardial tissue shows a polymorphic xanto-granulomatosous process, composed of vacuolated macrophages and lymphocytes, infiltrating normal myocardial fibers (A–C); (A) Hematoxylin & Eosin staining; (B) CD68 staining is expressed by foamy macrophages; (C) 5A8 staining, directed against the NH_2 terminal vasostatin-1 domain of CgA, shows a granular staining of myocyte cytoplasm. In (D) 5A8 staining on cross-section of myocardial fibers at higher magnification.

patients,[35,36] but this method can be obviously applied only to patients carrying identified mutations, and only to monitor the impact of a drug on mutated histiocytes.

We show here that both sTNF-Rs and CgA levels parallel response to therapy in individual patients, irrespective of the ongoing treatment. These factors, however, are possibly

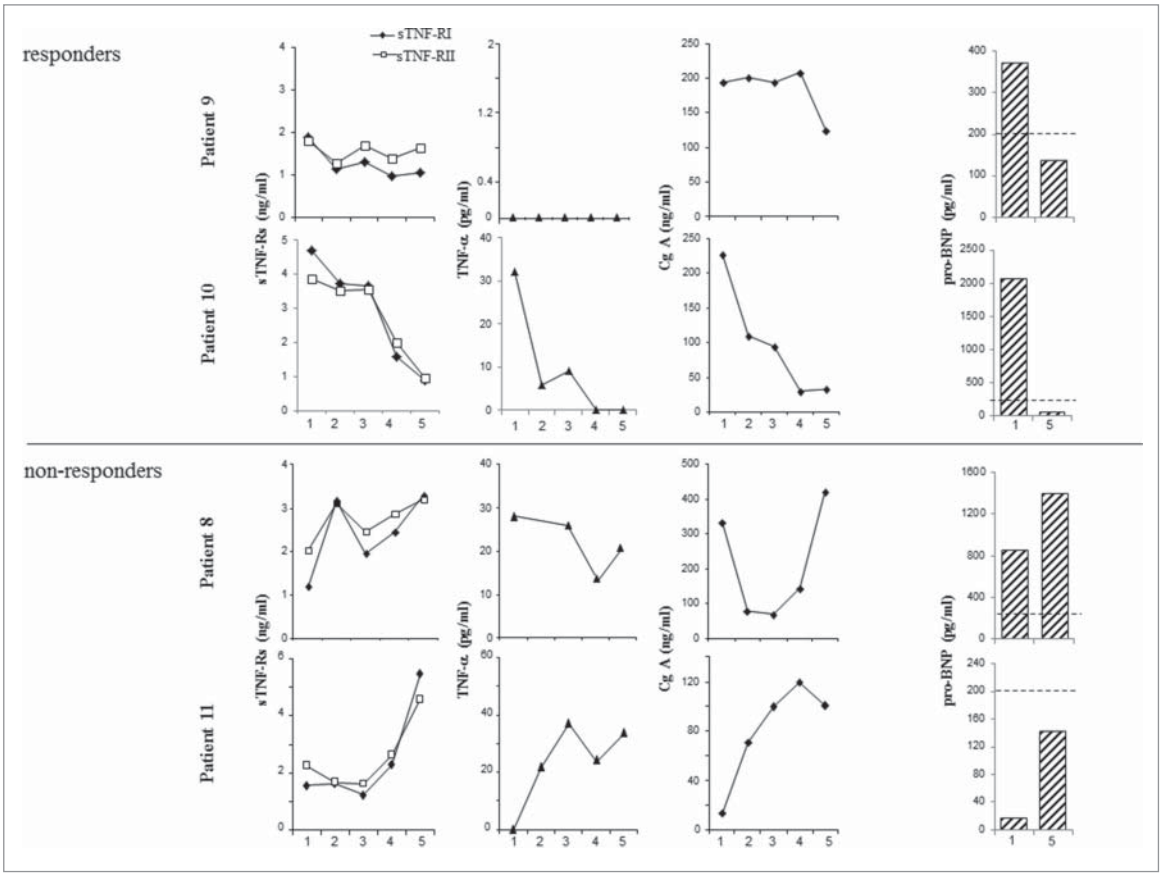


Figure 3. Kinetics of plasma sTNF-Rs and CgA parallel response to therapy in ECD patients. Plasma levels of sTNF-RI, sTNF-RII, TNF-α, and CgA were serially measured in four patients treated with TCZ (Patients 8 and 11) or IFX (Patients 9 and 10) and showing either response to therapy or disease progression. In the *x*-axis, time points corresponding to 3–6 mo intervals. Pro-BNP levels were also determined at the baseline (point 1) and at the end of the observation (point 5); dotted lines represent the cut-off value (200 ng/mL) for healthy age-matched males.

endowed with different clinical implications, the former being markers of ECD-associated inflammation, while the latter, together with pro-BNP, being specifically marker of ECD-associated cardiovascular involvement. Circulating CgA is a well-established marker of neuro-endocrine tumors and is emerging as a diagnostic/prognostic indicator of acute coronary syndromes and CHF.[34] Given that cardiovascular involvement in ECD is prognostically significant but often asymptomatic, our data suggest that CgA can be also exploited as an independent biomarker for the disease.

Patients and methods

Patients

We enrolled 37 patients with ECD, diagnosed on the basis of clinical imaging and histopathological criteria, including the accumulation inside lesions of $CD68^+$ $CD1a^-$ foamy histiocytes.[2-6] Seventeen patients were followed in the Unit of Medicine and Clinical Immunology of the OSR (Milan, Italy) and 20 in the Department of Internal Medicine and French Reference Center for Rare Autoimmune and Systemic Diseases of the Pitié-Salpêtrière Hospital (Paris, France). The study was approved by the locally appointed Ethics Committees in accordance with the Declaration of Helsinki and written informed consent was obtained from all subjects. Twenty age- and sex-matched healthy volunteers were enrolled as controls. In four patients samples were subsequently obtained every 3–6 mo after the baseline; two patients (Patients 9 and 11) were in treatment with the IL-6 receptor inhibitor tocilizumab (TCZ) and two (Patients 8 and 10) with the anti-TNF-α antibody infliximab (IFX) as second-line therapies because of contraindications or unresponsiveness to IFN-α treatment. Clinical evaluation, including collection of laboratory and radiological data (total-body computed tomography-CT-scan, Technetium-99m methylene diphosphonate-99^mTc-MDP-bone-scan, fluorine-18-2-fluoro-d-glucose positron emission tomography–FDGPET, brain and cardiac Magnetic Resonance Imaging–MRI) was performed at the baseline and repeated at the time of each blood collection.

Determination of TNF-α, sTNF-Rs, and CgA concentrations

Plasma levels of TNF-α were determined by the Bio-Plex Multiple-Cytokines Assay (Bio-Rad, Hercules, CA).[14] Plasma sTNF-Rs and CgA were measured by ELISA as described.[14,23]

Determination of pro-BNP concentration

Pro-BNP plasma levels were determined through a specific electrochemiluminescence (ECL) method (Elecsys® NT-proBNP, Roche) and analyzed with a Hitachi Cobas E601 analyzer.

Immunohistochemistry

Immunohistochemistry for CgA was performed using the mouse anti-human CgA monoclonal antibody 5A8, directed against the N-terminal domain (vasostatin-1) on paraffin sections of a myocardial sample.[27] Infiltrating histiocytes were identified by staining with an anti-human CD68 monoclonal antibody (DAKO, Glostrup, Denmark).[10]

Statistical analysis

Data are presented as mean ± SEM. We used the Mann–Whitney U test to compare parameters between ECD patients and controls. The correlation between CgA levels, TNF-Rs and pro-BNP in ECD patients was calculated by Spearman correlation analysis and is expressed as r coefficient. A p value below 0.05 was considered significant.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

Acknowledgments

The authors thank Kathy Brewer and the ECD Global Alliance (De Ridder, LA) for their continuous activities for patients with Erdheim–Chester disease and their unceasing support. We also thank Dr Barbara Vergani (Consorzio MIA) for histological analyses; Dr Giliola Calori (San Raffaele Scientific Institute) for her assistance in statistical analyses; and Dr Andrea Motta (San Raffaele Scientific Institute) for pro-BNP determination.

Funding

The work was supported in part by a Grant from the Italian Ministry of Health (GR 2009-1594586 to LD) and by a grant from ECD Global Alliance (to MF & LD).

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