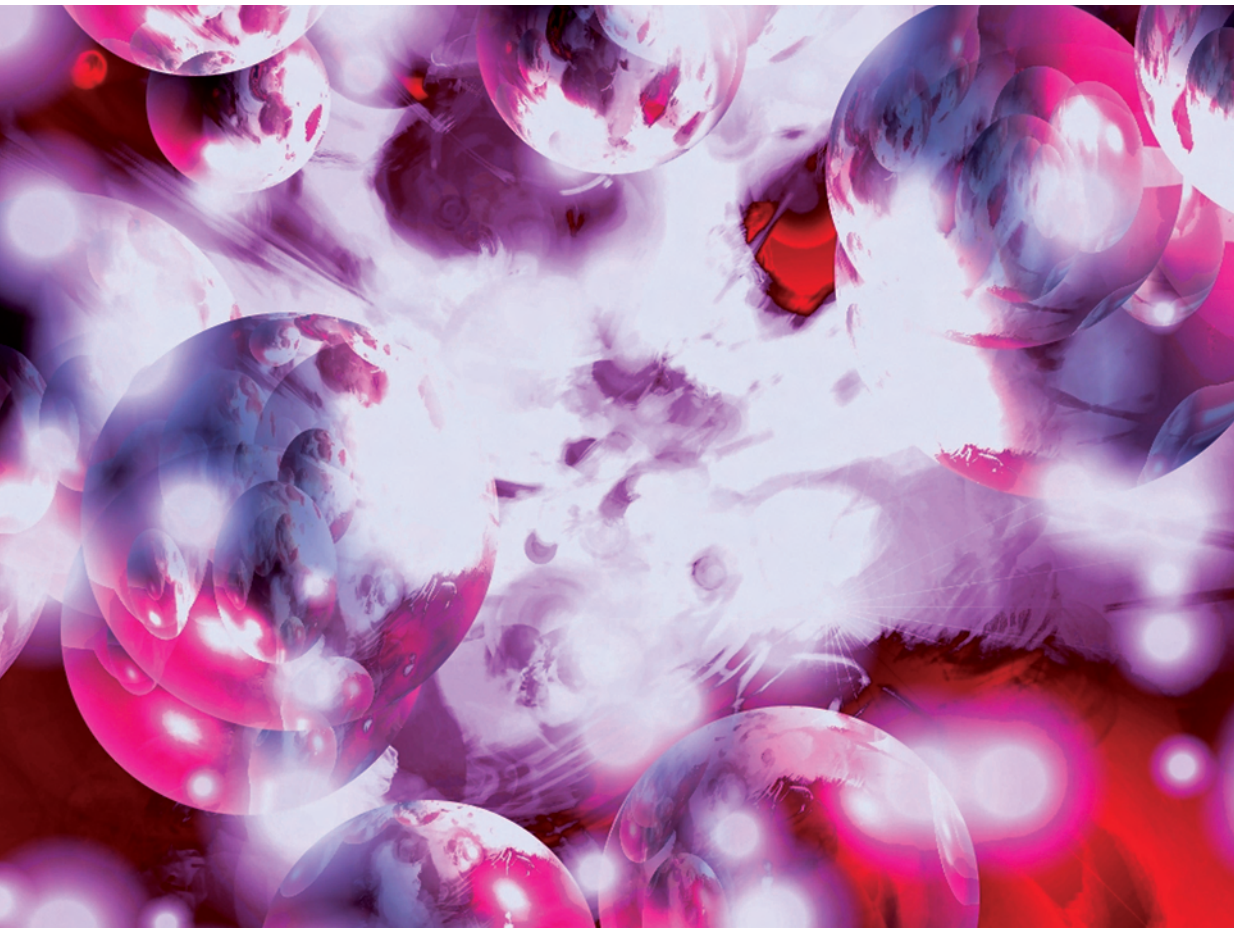
“Inflammatory Soup”

Mediators of inflammation in CRPS



Feikje Wesseldijk

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Chapter 1
Introduction

Introduction

Complex Regional Pain Syndrome type 1 (CRPS1) is a disease of the extremity that usually occurs as a complication after surgery or trauma, although spontaneous occurrence has also been described [1]. The clinical features include pain/sensory abnormalities, vasomotor dysfunction, edema/sudomotor dysfunction, and motor/trophic changes [2]. The diagnosis of CRPS is based on findings during the history and physical examination. Several diagnostic criteria sets have been developed, and the most used are the Veldman criteria [3], the IASP criteria (the International Association for the Study of Pain) [4], and the Bruehl criteria [5, 6]. There is a distinction between CRPS types 1 and 2. Type 1 occurs without any peripheral nerve lesion, whereas in type 2 there is definite peripheral nerve damage.

In the Netherlands, the incidence of CRPS is estimated to be approximately 26.2 per 100,000 person years, with a median age of onset of 52.7 years. CRPS occurs more often in females than in males, with a ratio of approximately 3.4:1. The upper extremity is more often affected than the lower extremity, and the right and the left sides of the body are affected with the same frequency. A fracture is the most common precipitating event (44%) for CRPS, followed by a contusion/sprain in 17% of the population [7].

There is a long-standing belief described in the literature that untreated CRPS develops through a sequence of distinct stages, each stage characterized by a different pattern of signs and symptoms [3, 8-10]. While the clinical descriptions of these hypothesized stages have differed somewhat in the details among authors, most conform to the general description by Bonica (1990). Although only limited empirical tests of this hypothesized staging of CRPS have been reported, this concept has been frequently accepted as a fact in the CRPS literature (e.g., [8, 10]).

The early, acute stage of CRPS (Stage I) is believed to be characterized by pain/sensory abnormalities (e.g., hyperalgesia, allodynia), signs of vasomotor dysfunction that affect skin color, often increased temperature of the skin, prominent edema, and sudomotor disturbance. Stage II (dystrophic stage) is characterized by more marked pain/sensory dysfunction, continued evidence of vasomotor dysfunction and sudomotor dysfunction, with development of significant motor/trophic changes. Stage III (atrophic stage) is characterized by decreased pain/sensory disturbance, continued vasomotor disturbance often with a decreased

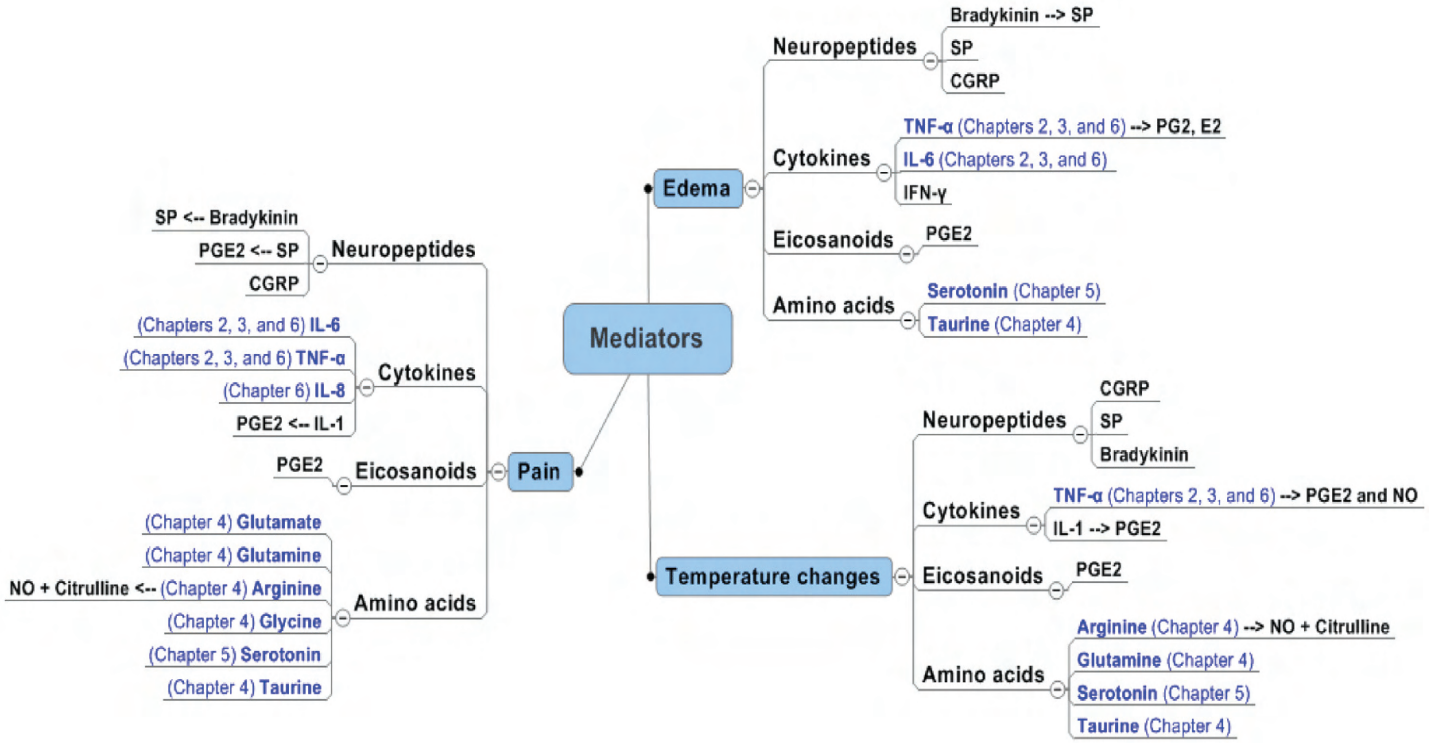

Figure 1. Mediators that might be responsible for the clinical signs and symptoms of CRPS.
SP, substance P; CGRP, calcitonin-gene related peptide; TNF-α, tumor necrosis factor alpha; IL-1, interleukin-1; IL-6, interleukin-6; IL-8, interleukin-8; PGE2, prostaglandin E2; NO, nitric oxide.

temperature of the skin, and markedly increased motor/trophic changes that become irreversible [2, 11, 12].
Because the prominent signs and symptoms seen in the early stage of CRPS resemble the characteristic signs of inflammation, such as pain, increased skin temperature and skin color changes, swelling of the extremity, and loss of function, the question that arises is whether or not an inflammatory process is involved in CRPS. An inflammatory process is substantiated by the generation of mediators of inflammation at the site of the injury, a specific antagonist or synthesis inhibitor that shows a beneficial effect, and a worsening of the clinical manifestations by injection of the mediator(s). Figure 1 depicts some candidate mediators for one or more signs and symptoms seen in CRPS. Their common functions are described below.

Neuropeptides
Bradykinin is an endogenous peptide produced in plasma and peripheral tissue that can activate two different receptors (B1 and B2). The B2 receptors are found both peripherally and centrally and are responsible for the physiological response of bradykinin. However, B1 receptors can be activated under pathological conditions or by trauma, and also by endotoxins and cytokines. Bradykinin increases pain sensitivity via a glutamate-dependent activation of the N-methyl-aspartate (NMDA) receptor [13]. Bradykinin can provoke substance P (SP) release, and both can trigger mast cell secretion [14]. They may also be involved in the development of inflammatory responses. The mast cells release vasodilatory, nociceptive, and pro-inflammatory mediators, thus causing an allergic reaction [15].
The sensory system stores SP and calcitonin gene-related peptide (CGRP). SP is a co-transmitter in primary afferent nerve fibers, involved in nociception in both the peripheral nervous system and the central nervous system. It is responsible for neurogenic inflammation, including local cell response, skin reddening, edema formation, and mechanical hyperalgesia. It is suggested that SP acts by release of inflammatory mediators, such as cytokines, oxygen radicals, arachidonic acid, and histamine. It also enhances lymphocyte proliferation and immunoglobulin production [16, 17]. By activation of the NK1 receptor, SP increases the production of prostaglandin E2 (PGE2) and evokes thermal hyperalgesia [14]. SP mediates mainly plasma leakage (extravasation), and its

levels are increased during stress and inflammation [15], whereas CGRP is the major vasodilator neurotransmitter in the coronary, cutaneous, and cerebral microvasculature [15, 17, 18].
Just like SP, CGRP plays an important role in the development of inflammatory pain and hyperalgesia in primary afferent neurons. Both CGRP (via the CGRP receptor) and SP (via the NK1 receptor) also act via different mechanisms to induce vasodilation [14, 15, 19].

Cytokines
Interleukin-1 (IL-1) is produced by chondrocytes and other cells in the joints and plays an important role in cartilage degeneration by stimulating the synthesis of degenerative enzymes that inhibit the production of proteoglycans. Tumor necrosis factor α (TNF-α), and IL-6 appear to act synergistically with IL-1 and are all found in inflamed joints, along with SP and IL-1β [15]. IL-1 and IL-6 are increased during stress or inflammation [15].
IL-6 is responsible for osteoporosis and induces hypersensitivity to pain and also fatigue and depression [15]. Release of TNF-α and IL-6 from monocytes/macrophages provokes neuropathic pain. IL-8 also promotes sympathetic pain [15]. The level of IL-1β is significantly correlated with the degree of pain (hyperalgesia), as well as TNF-α levels [15].
TNF-α may be a key player in the mediation of mechanical hyperalgesia in neuropathic pain. Animal studies support a role for TNF-α in mechanical hyperalgesia. TNF-α can provoke ectopic nerve activity and neutralizing antibodies to the TNF receptor-1 and reduce hyperalgesia in animals. In humans, elevated levels of soluble TNF receptor-1 (sTNF-R1) have been linked to allodynia in polyneuropathies. TNF-α can promote neurogenic inflammation and neuropeptide release from nerve terminals and is more involved in sensitization processes within the nociceptive system than in directly provoking pain [20].
Interferon-gamma (IFN-γ) is a proinflammatory cytokine that is mainly released by T-lymphocytes and natural killer cells. It plays a pivotal role in the pathology of inflammatory diseases in the central nervous system, such as multiple sclerosis. It directly induces neuronal dysfunction and enhances glutamate neurotoxicity mediated via the alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic (AMPA) receptor, not via the NMDA-receptor. One study provides findings for a novel mechanism of neuronal

excitotoxity, which may occur in both inflammatory and neurodegenerative diseases in the central nervous system [21].

Eicosanoids

Arachidonic acid is released by phospholipase A2 from phospholipids. The prostanoids are metabolized into biologically active prostaglandins (PGs). Prostaglandin E2 (PGE2) is the principal proinflammatory prostanoid and contributes, in particular, to the two key features of inflammation, pain hypersensitivity and temperature changes. At the site of inflammation, PGE2 sensitizes peripheral nociceptors through activation of EP receptors present on sensory neurons, the phenomenon of peripheral sensitization. PGE2 is also produced in the spinal cord after tissue injury, where it contributes to central sensitization, an increase in the excitability of spinal dorsal horn neurons that produces pain hypersensitivity. PGE2, when produced at the site of tissue injury and inflammation, acts on capillaries to produce vasodilatation and edema and on macrophages to stimulate cytokine release [22].
By activation of the NK1 receptor, SP increases the production of PGE2 and evokes thermal hyperalgesia [14]. IL-1 also stimulates the production of PGE2, contributing to vasodilation and pain. Following the release of TNF-α, there is an increase in the formation of PGE2, which can lead to edema formation by regulation of the vascular tone [15, 18].

Amino acids

A number of amino acids play an important role in the transmission of pain. As described above, bradykinin increases sensitization of pain via the NMDA receptors in the central nervous system by glutamate. Together with IFN-γ, glutamate contributes to neurotoxicity [21].
Levels of pain intensity are related to spinal fluid levels of arginine. Increased plasma arginine levels could result in vasoconstriction, diminished tissue blood distribution, and spread of pain [23]. Arginine is a precursor to nitric oxide (NO) and citrulline [15] and the citrulline/arginine ratio is regarded as an index of NO synthesis [24-26]. NO is also generated by macrophages and neutrophils as part of the human immune response [27].
The NO system has been hypothesized to be involved in depression. NO has been shown to modulate the effects of monoaminergic neurotransmitters such as serotonin, noradrenaline, and dopamine

and is thought to be involved in the pathogenesis of depression [24]. IFN-γ enhances the production of TNF-α and NO. Subsequent to the release of TNF-α, there is an increase in the formation of NO, which can lead to edema formation by regulation of the vascular tone [15, 18]. NO can modulate CGRP release and synthesis [28] and may also contribute to IL-1–induced degeneration of cartilage [15].

The enzyme glutaminase converts glutamine into glutamate. Furthermore, glutamine is used for the synthesis of urea in the liver, for renal ammoniagenesis, and for gluconeogenesis in both liver and kidney and as a major respiratory fuel for many cells [24, 29]. Glutamine inhibits the generation of arginine in cultured endothelial cells [30].

Glycine is a co-agonist of the NMDA receptor and is known to modulate immune cell responses in which the inhibitory effects on macrophages could be important [31]. It is mainly responsible for neuropathic pain, which manifests as allodynia and hyperalgesia [32].

The neurotransmitter serotonin (5-HT) is involved in many psychiatric disorders, including panic disorder, suicidal behavior, and depression. Plasma 5-HT has been suggested as a peripheral indicator of central serotonergic activity. Most of the 5-HT is stored in thrombocytes [33]. In blood vessels, 5-HT evokes vasoconstriction through activation of the 5-HT2A receptor. Important actions and functions of 5-HT are vascular constriction and contraction of other smooth muscles, increased microvascular permeability, induction of blood platelet aggregation, stimulation of peripheral nociceptive nerve endings, and excitation/inhibition of neurons in the central nervous system. Clinical conditions associated with disturbed 5-HT function include migraine, carcinoid syndrome, mood disorders, and anxiety [34, 35]. Moreover, platelet 5-HT release is inhibited by NO [36].

Taurine is found in high concentrations in muscle, brain, heart, and blood. This suggested that it is suggested that it may play an important role in neuromodulation, osmoregulation, and thermoregulation. Antihypertensive effects of taurine have been demonstrated in several experimental models. Several rat models suggest that taurine plays an important role in the maintenance and regulation of vascular tone in normal and pathological conditions [37]. It has been reported that taurine reduces cyclic GMP, which evokes vasodilation [38]. The release of taurine in various

cell-damaging conditions, including ischemia and increased free radical production, has been shown [39]. Taurine in turn has antioxidative properties [40]. Very recently, it has been shown that taurine can diminish neuropathic nociception, possibly through interaction with the glycine receptor [41].

Hypothesis and Aim

Our hypothesis is that a variety of mediators of inflammation are present in tissue fluid of CRPS1 patients in the initial stage of the disease, causing the clinical signs and symptoms seen in CRPS1, which resemble the characteristics of inflammation. Furthermore, it was hypothesized that the inflammatory process would diminish over time by normalization of these mediators of inflammation.

The aim of this thesis was to measure these potential mediators in the plasma and fluid of artificially induced skin blisters of CRPS1 patients. Furthermore, we sought to determine whether these mediators are responsible for the characteristics of CRPS1 and to characterize the relationship between cytokine levels in plasma and blister fluid and duration of the disease. The following disease characteristics were assessed: pain, differences in temperature, volume, and mobility between the extremities, and psychological dysfunction.

References

1. Janig, W. and R. Baron,*Complex regional pain syndrome: mystery explained?* Lancet Neurol, 2003. 2(11): p. 687-97.
2. Bruehl, S., R.N. Harden, B.S. Galer, S. Saltz, M. Backonja, and M. Stanton-Hicks, *Complex regional pain syndrome: are there distinct subtypes and sequential stages of the syndrome?* Pain, 2002. 95(1-2): p. 119-24.
3. Veldman, P.H., H.M. Reynen, I.E. Arntz, and R.J. Goris, *Signs and symptoms of reflex sympathetic dystrophy: prospective study of 829 patients.*Lancet, 1993. 342(8878): p. 1012-6.
4. Stanton-Hicks, M., W. Janig, S. Hassenbusch, J.D. Haddox, R. Boas, and P. Wilson, *Reflex sympathetic dystrophy: changing concepts and taxonomy. Pain,* 1995. 63(1): p. 127-33.
5. Harden, R.N., S. Bruehl, B.S. Galer, S. Saltz, M. Bertram, M. Backonja, R. Gayles, N. Rudin, M.K. Bhugra, and M. Stanton-Hicks, *Complex regional pain syndrome: are the IASP diagnostic criteria valid and sufficiently comprehensive?* Pain, 1999. 83(2): p. 211-9.
6. Bruehl, S., R.N. Harden, B.S. Galer, S. Saltz, M. Bertram, M. Backonja, R. Gayles, N. Rudin, M.K. Bhugra, and M. Stanton-Hicks, *External validation of IASP diagnostic criteria for Complex Regional Pain Syndrome and proposed research diagnostic criteria. International Association for the Study of Pain.* Pain, 1999. 81(1-2): p. 147-54.
7. de Mos, M., A.G. de Bruijn, F.J. Huygen, J.P. Dieleman, B.H. Stricker, and M.C. Sturkenboom, *The incidence of complex regional pain syndrome: a population-based study.*Pain, 2007. 129(1-2): p. 12-20.
8. Bonica, J.J., *Evolution and current status of pain programs.* J Pain Symptom Manage, 1990. 5(6): p. 368-74.
9. Schwartzman, R.J. and J. Kerrigan, *The movement disorder of reflex sympathetic dystrophy.* Neurology, 1990. 40(1): p. 57-61.
10. Schwartzman, R.J. and T.L. McLellan, *Reflex sympathetic dystrophy.* A review. Arch Neurol, 1987. 44(5): p. 555-61.
11. Munnikes, R.J., C. Muis, M. Boersma, C. Heijmans-Antonissen, F.J. Zijlstra, and F.J. Huygen, *Intermediate stage complex regional pain syndrome type 1 is unrelated to proinflammatory cytokines.* Mediators Inflamm, 2005(6): p. 366-72.
12. Huygen, F.J., S. Niehof, J. Klein, and F.J. Zijlstra, *Computer-assisted skin videothermography is a highly sensitive quality tool in the diagnosis and monitoring of complex regional pain syndrome type I.* Eur J Appl Physiol, 2004. 91(5-6): p. 516-24.
13. Wang, H., T. Kohno, F. Amaya, G.J. Brenner, N. Ito, A. Allchorne, R.R. Ji, and C.J. Woolf, *Bradykinin produces pain hypersensitivity by potentiating spinal cord glutamatergic synaptic transmission.* J Neurosci, 2005. 25(35): p. 7986-92.
14. Tang, H.B., Y.S. Li, K. Arihiro, and Y. Nakata, *Activation of the neurokinin-1 receptor by substance P triggers the release of substance P from cultured adult rat dorsal root ganglion neurons.*Mol Pain, 2007. 3(1): p. 42.
15. Omoigui, S., *The biochemical origin of pain: the origin of all pain is inflammation and the inflammatory response. Part 2 of 3 - inflammatory profile of pain syndromes.* Med Hypotheses, 2007. 69(6): p. 1169-78.

16. Gradl, G., B. Finke, S. Schattner, P. Gierer, T. Mittlmeier, and B. Vollmar, *Continuous intra-arterial application of substance P induces signs and symptoms of experimental complex regional pain syndrome (CRPS) such as edema, inflammation and mechanical pain but no thermal pain.* Neuroscience, 2007. 148(3): p. 757-65.
17. Leis, S., M. Weber, M. Schmelz, and F. Birklein, *Facilitated neurogenic inflammation in unaffected limbs of patients with complex regional pain syndrome.* Neurosci Lett, 2004. 359(3): p. 163-6.
18. Starr, A., R. Graepel, J. Keeble, S. Schmidhuber, N. Clark, A. Grant, A.M. Shah, and S.D. Brain, *A reactive oxygen species-mediated component in neurogenic vasodilatation.* Cardiovasc Res, 2008.
19. Greco, R., C. Tassorelli, G. Sandrini, P. Di Bella, S. Buscone, and G. Nappi, *Role of calcitonin gene-related peptide and substance P in different models of pain.* Cephalalgia, 2008. 28(2): p. 114-26.
20. Maihofner, C., H.O. Handwerker, B. Neundorfer, and F. Birklein, *Mechanical hyperalgesia in complex regional pain syndrome: a role for TNF-alpha?* Neurology, 2005. 65(2): p. 311-3.
21. Mizuno, T., G. Zhang, H. Takeuchi, J. Kawanokuchi, J. Wang, Y. Sonobe, S. Jin, N. Takada, Y. Komatsu, and A. Suzumura, *Interferon-{gamma} directly induces neurotoxicity through a neuron specific, calcium-permeable complex of IFN-{gamma} receptor and AMPA GluR1 receptor.* Faseb J, 2008.
22. Lin, C.R., F. Amaya, L. Barrett, H. Wang, J. Takada, T.A. Samad, and C.J. Woolf, *Prostaglandin E2 receptor EP4 contributes to inflammatory pain hypersensitivity.* J Pharmacol Exp Ther, 2006. 319(3): p. 1096-103.
23. Groeneweg, J.G., F.J. Huygen, C. Heijmans-Antonissen, S. Niehof, and F.J. Zijlstra, *Increased endothelin-1 and diminished nitric oxide levels in blister fluids of patients with intermediate cold type complex regional pain syndrome type 1.* BMC Musculoskelet Disord, 2006. 7: p. 91.
24. Fekkes, D., M. Bannink, W.H. Kruit, A.R. Van Gool, P.G. Mulder, S. Sleijfer, A.M. Eggermont, and G. Stoter, *Influence of pegylated interferon-alpha therapy on plasma levels of citrulline and arginine in melanoma patients.* Amino Acids, 2007. 32(1): p. 121-6.
25. Dewanjee, M.K., *Molecular biology of nitric oxide synthases. Reduction of complications of cardiopulmonary bypass from platelets and neutrophils by nitric oxide generation from L-arginine and nitric oxide donors.* Asaio J, 1997. 43(3): p. 151-9.
26. Jugdutt, B.I., *Nitric oxide and cardioprotection during ischemia-reperfusion.* Heart Fail Rev, 2002. 7(4): p. 391-405.
27. Janaway, C., P. Travers, M. Walport, and M. Shlomchik, 2005. *Immunobiology: the immune system in health and disease.* 0-8153-4101-6.
28. Ghatta, S. and N. D, *Calcitonin gene-related peptide: Understanding its role.* Indian J Pharmacol 2004. 36: p. 277-283.
29. Curthoys, N.P. and M. Watford, *Regulation of glutaminase activity and glutamine metabolism.* Annu Rev Nutr, 1995. 15: p. 133-59.
30. Sessa, W.C., M. Hecker, J.A. Mitchell, and J.R. Vane, *The metabolism of L-arginine and its*

significance for the biosynthesis of endothelium-derived relaxing factor: L-glutamine inhibits the generation of L-arginine by cultured endothelial cells. Proc Natl Acad Sci U S A, 1990. 87(21): p. 8607-11.

31. Schilling, T. and C. Eder, *A novel physiological mechanism of glycine-induced immunomodulation: Na+-coupled amino acid transporter currents in cultured brain macrophages.* J Physiol, 2004. 559(Pt 1): p. 35-40.
32. Jorum, E., T. Warncke, and A. Stubhaug, *Cold allodynia and hyperalgesia in neuropathic pain: the effect of N-methyl-D-aspartate (NMDA) receptor antagonist ketamine--a double-blind, cross-over comparison with alfentanil and placebo.* Pain, 2003. 101(3): p. 229-35.
33. Fekkes, D., L. Timmerman, and L. Pepplinkhuizen, *Effects of clomipramine on plasma amino acids and serotonergic parameters in panic disorder and depression.* Eur Neuropsychopharmacol, 1997. 7(3): p. 235-9.
34. Houston, D.S. and P.M. Vanhoutte, *Serotonin and the vascular system. Role in health and disease, and implications for therapy.* Drugs, 1986. 31(2): p. 149-63.
35. Roberts, M.H., *Involvement of serotonin in nociceptive pathways.* Drug Des Deliv, 1989. 4(2): p. 77-83.
36. Juhasz, G., T. Zsombok, E.A. Modos, S. Olajos, B. Jakab, J. Nemeth, J. Szolcsanyi, J. Vitrai, and G. Bagdy, *NO-induced migraine attack: strong increase in plasma calcitonin gene-related peptide (CGRP) concentration and negative correlation with platelet serotonin release.* Pain, 2003. 106(3): p. 461-70.
37. Niu, L.G., M.S. Zhang, Y. Liu, W.X. Xue, D.B. Liu, J. Zhang, and Y.Q. Liang, *Vasorelaxant effect of taurine is diminished by tetraethylammonium in rat isolated arteries.* Eur J Pharmacol, 2008. 580(1-2): p. 169-74.
38. Hilgier, W., E. Anderzhanova, S.S. Oja, P. Saransaari, and J. Albrecht, *Taurine reduces ammonia- and N-methyl-D-aspartate-induced accumulation of cyclic GMP and hydroxyl radicals in microdialysates of the rat striatum.* Eur J Pharmacol, 2003. 468(1): p. 21-5.
39. Saransaari, P. and S.S. Oja, *Characteristics of taurine release induced by free radicals in mouse hippocampal slices.* Amino Acids, 2004. 26(1): p. 91-8.
40. Zhang, M., I. Izumi, S. Kagamimori, S. Sokejima, T. Yamagami, Z. Liu, and B. Qi, *Role of taurine supplementation to prevent exercise-induced oxidative stress in healthy young men.* Amino Acids, 2004. 26(2): p. 203-7.
41. Pellicer, F., A. Lopez-Avila, U. Coffeen, J. Manuel Ortega-Legaspi, and R.D. Angel, *Taurine in the anterior cingulate cortex diminishes neuropathic nociception: a possible interaction with the glycine(A) receptor.*
    Eur J Pain, 2007. 11(4): p. 444-51.

Chapter 2

Tumor necrosis factor-α and interleukin-6 are not correlated with the characteristics of CRPS type 1 in 66 patients

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Abstract
It was previously shown in a group of 9 patients with Complex Regional Pain Syndrome type 1 (CRPS1) that levels of the proinflammatory cytokines tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) are higher in blister fluid from the involved side. We hypothesize that local inflammation is responsible for the characteristics of CRPS1. The aim of this study was to confirm the previous observation in a large group of CRPS1 patients, repeating the measurement of TNF-α and IL-6 in blister fluid. Furthermore, we sought to determine whether these cytokines are responsible for the characteristics of CRPS1 and characterize the relationship between cytokine levels and duration of the disease. Sixty-six patients with CRPS1 participated. Skin blisters were artificially induced for measurement of cytokines in both extremities. The following disease characteristics were assessed: pain and differences in temperature, volume, and mobility between the extremities. TNF-α and IL-6 levels were significantly higher in blister fluid from the involved side. However, cytokine levels did not correlate with the characteristics or duration of the disease. Our findings confirm the presence of local inflammation in a population of 66 patients in the first 2 years of CRPS1. Proinflammatory cytokines seem to be only partly involved in the pathophysiology of CRPS1, as indicated by the lack of coherence between TNF-α and IL-6 levels and the signs and symptoms of inflammation and disease duration. Other inflammatory mediators and mechanisms, such as central sensitization, are probably involved in the early stages of CRPS1.

Introduction
Complex Regional Pain Syndrome type 1 (CRPS1) is a disease of the extremity that usually occurs as a complication after surgery or trauma, although spontaneous occurrence has also been described [1].
In a recent epidemiological study in the Netherlands, the incidence of CRPS1 for fractures was estimated to be approximately 26.2 per 100,000 person years, with a median age of onset of 52.7 years. CRPS1 occurs more often in females than in males, the ratio being approximately 3.4:1. Whereas the upper extremity is more often affected than the lower extremity, the right and the left sides of the body are affected with the same frequency [2].
The pathophysiology of CRPS1 remains a matter of debate. In

general, three mechanisms are thought to be involved: afferent mechanisms (e.g. neurogenic inflammation) [3-5], efferent mechanisms (e.g. autonomic disturbances) [6], and central nervous system mechanisms (e.g. cerebral plasticity) [7]. Based on our review of the literature regarding CRPS pathophysiology, we hypothesize that after trauma or surgery, the normal sterile inflammatory response is exacerbated by a genetic and/or acquired immunologic disorder [8]. Neuroimmune activation of cells in the peripheral nervous system, which is part of the afferent mechanism, apparently results in central sensitization and exacerbation of pain [9]. Neuropeptides, cytokines, and other mediators are released during the inflammation [10, 11] and cause the prominent signs and symptoms, which resemble inflammation; these include increased skin temperature, edema, pain, loss of function, and redness [8, 12].

In our previous work involving 9 CRPS1 patients, we found higher levels of the initially produced proinflammatory cytokines tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) in artificially induced skin blisters on the involved side. These observations suggest local inflammation [10, 13]. The aim of this study was to confirm the previous observation by measuring TNF-α and IL-6 in blister fluid in a larger group of CRPS1 patients, all of whom fulfill the diagnostic criteria described by Bruehl et al. [14]. Furthermore, we sought to determine whether these cytokines cause the characteristic signs and symptoms of the syndrome and to investigate the correlation between cytokine levels and disease duration.

Methods

Patients

Sixty-six patients with CRPS1 in one extremity, as defined by the diagnostic criteria of Bruehl, participated [14]. Patients with other diseases that could explain the signs and symptoms were excluded. The patients participated in several studies conducted between 2001 and 2005 to investigate the pathophysiology of CRPS1 or the effects of specific treatments for CRPS1 [10, 13, 15, 16]. The protocol was approved by the Medical Ethics Committee of the Erasmus MC Rotterdam (MEC no. 1989.780/2001/24). Guidelines according to the Declaration of Helsinki (amended version of 2002) and Good Clinical Practice (ICH/GCP version 1996)

were followed. Data collection and calculations were performed according to guidelines for registration of personal data.
Data collected before the start of these treatments (T0) were used to describe the patients, so none of the treatments would affect the results of the measurements in the current study.

Pain assessment
Patients rated their pain intensity using a Visual Analogue Scale (VAS) ranging from 0-100 mm [17].

Temperature measurement
Skin temperature was measured using an infrared tympanic probe thermometer (First Temp Genius®; Sherwood Medical Crawley, Sussex, UK) [18]. Measurements were obtained on the dorsal aspect of the hand or foot in a matrix of five points. The difference in mean temperature between the involved and contralateral extremities was calculated.

Assessment of volume
Volume was measured with a volumeter, which measures the amount of water displaced by immersion of a body part [19]. The difference between the involved and contralateral extremities was calculated as a percentage of the contralateral extremity.

Assessment of mobility
Mobility was assessed by measuring the active range of motion (AROM), which is defined as the arc of motion requiring muscle power to achieve the motion of a joint. In the upper extremity, the AROM was measured for dorsal and palmar flexion in of the wrist, and for flexion and extension of the metacarpophalangeal and proximal interphalangeal joints of digits 1, 2, and 3 [20]. In the lower extremity, the AROM was measured for flexion and extension of the knee, ankle, and digit 1 of the foot, and inversion and eversion of the ankle [21].
The position of the patients and the method of measurement were standardized for each joint, in accordance with the clinical assessment recommendations of the American Society of Hand Therapists [22]. The AROM on the involved extremity was multiplied by 100 and divided by the AROM on the contralateral extremity to derive the percentage of normal mobility.

Blisters
Blisters were induced using a suction method [10, 15, 23]. A skin suction chamber was positioned on the skin of the involved and contralateral extremities. A vacuum of 300 mmHg was applied with an Atmoforte 350A aspirator pump (ATMOS Medizintechnik, Lenzkirch, Germany). After 15 min, the vacuum was reduced to 250 mmHg, and after another 15 min it was reduced to 200 mmHg. This negative pressure was maintained for 2-2.5 hours. The blisters created were punctured, and fluid was pooled from each side into a 1.5-ml Eppendorf conical polypropylene tube and centrifuged for 5 min at 1600 xg. The mean recovery of supernatants was 152 ± 103 (SD) µl for the involved side and 153 ± 99 µl for the contralateral side. All samples were stored in 1-ml conical polypropylene tubes at -80 °C until analysis [10, 23].

Cytokine assays
Blister fluid samples were diluted (mean dilution factor 4.1 ± 1.6) in appropriate calibrator diluent assay buffer for the direct measurement of cytokines. Cytokine assays were performed following the manufacturer's protocol (PeliKine™ human ELISA kits for IL-6 [M1906] and TNF-α [M1920]; CLB, Amsterdam, the Netherlands). The standard curve ranges and mean calculated zero signal ± 3 (SD) were 0-80 and 0.3 pg/ml for IL-6 and 0-1000 and 1 pg/ml for TNF-α. The absorbance per well was measured at 450 nm with a Medgenix EASIA reader. Sample concentrations were calculated using the appropriate standard calibration lines and the Softmax® software of the reader.

Statistical analysis
Data were analyzed using SPSS for Windows, version 14.0. The nonlinear distributed data of cytokine content in blister fluid and the objective parameters temperature, volume, and AROM were described as median and ranges. The linear distributed data of the subjective parameter pain was described as the median and SD. Paired samples were compared using the Wilcoxon signed ranks test, and correlations were analyzed using Spearman's rank correlation test.

Table 1. Characteristics of the study population

Patient characteristic	Value
Gender: male/female	16/50
Age in years	48 (21-78)
Duration of CRPS1, months	7 (1-168)
Location: upper/lower extremity	50/16
Side: right/left	34/32
Cause: trauma (fracture, accident)/surgery/ spontaneous/other	40 (25, 15)/17/2/7

Data are presented as the n or median (range)

Results

Patient characteristics are presented in Table 1. All 66 patients reported continuing pain that was disproportionate to the inciting event. For the sensory category of the Bruehl criteria set, 100% reported an abnormality in sensation of the extremity. Similarly, for the vasomotor category, sudomotor/edema category, and motor/trophic category, 100% of the population reported an abnormality for the involved side. At physical examination, 76% of the patients had a difference in sensation of the skin, 98% had a difference in vasomotor signs between sides, 94% had a difference in the sudomotor/edema category between sides, and 94% had an abnormality in the motor/trophic category. Table 2 lists all signs and symptoms for the four categories separately.

The signs and symptoms of impairment were assessed in terms of the subjective parameter pain and the objective parameters temperature, volume, and mobility. Patients reported a VAS score with a median of 55 mm (SD 22). In the first 2 years of the disease, 41% of the population had a warm extremity (median difference between involved and contralateral side 0.5 ºC, range 0.02 to 4.1), whereas 56% had a cold extremity (median -1.0 ºC, range -4.8 to -0.02). Three percent of patients did not show any difference in temperature. Approximately the same distribution was found for edema (39% of the patients; median difference 5.9% of the contralateral side, range 0.8 to 10.4) and atrophy (59% of the patients; median difference -0.8% of the contralateral side, range -0.3 to -1.1). In 95% of the patients, mobility was lower for the involved versus contralateral side (median 69.7% of the contralateral side, range 0.3% to 97.7%), whereas in 5% of the patients, mobility of the involved extremity was better (median 103.8% of the contralateral side, range 101.3 to 121.7).

Table 2. Characteristic signs and symptoms of the study population

Criteria of Bruehl		Reported by patient	Displayed by clinician
Continuing pain		100 %	
Sensory	Total	100%	76%
	Allodynia	68 %	60 %
	Hyperesthesia	92 %	34 %
Vasomotor	Total	100%	98%
	Temperature asymmetry / changes	98 %	87 %
	Skin color asymmetry / changes	98 %	77 %
Sudomotor / oedema	Total	100%	94%
	Oedema	96 %	87 %
	Sweating asymmetry / changes	68 %	38 %
Motor / trophic	Total	100%	94%
	Decreased range of motion	94 %	92 %
	Weakness	75 %	28 %
	Tremor / myoclonus / dystonia	62 %	26 %
	Hair growth asymmetry / changes	38 %	25 %
	Nail growth asymmetry / changes	32 %	9 %
	Skin changes	11 %	13 %

In 57 of the 66 patients, skin blisters were successfully induced. Levels of TNF-α and IL-6 were significantly higher in the blister fluid from the involved versus contralateral extremity (Table 3). For 74% of the patients, the levels of TNF-α were elevated on the involved side (median difference between the involved and contralateral side 23 pg/ml, range 1 to 718 pg/ml). For 25% of the patients, the levels of TNF-α were lower on the involved side (median difference between the involved and contralateral side -18 pg/ml, range -295 to -1 pg/ml). For 1% of patients, no difference was found. For 84% of the patients, the levels of IL-6 were elevated on the involved side (median difference between the involved and contralateral side 44 pg/ml, range 1 to 626 pg/ml). In 12% of the patients, the levels of IL-6 were lower on the involved side (median difference between the involved and contralateral side -8 pg/ml, range -258 to -2 pg/ml). In 4%, no difference was found between the two sides.

There was a significant correlation between the differences in TNF-α and IL-6 between sides (Spearman's correlation coefficient = 0.79; $p < 0.001$). There was no significant correlation between the cytokines and typical characteristics of inflammation such as pain, heat, edema, and loss of function or duration of the disease.

Table 3. Data on TNF-α and IL-6 levels in blister fluid in the involved and contralateral extremities.

	Involved side	Contralateral side
TNF-α (pg/ml)	28 (1–795)*	14 (1– 616)
IL-6 (pg/ml)	43 (1– 662)*	3 (1– 434)

Data are presented as median (range)

* Wilcoxon signed-ranks test $P < 0.001$

Discussion

In 66 CRPS1 patients in the first two years of the disease, levels of the proinflammatory cytokines TNF-α and IL-6 were significantly higher in the involved versus contralateral extremity. These findings are in agreement with earlier results found in a group of nine CRPS1 [10, 15].

In an earlier study, we found high levels of the proinflammatory cytokines IL-6 and TNF-α in suction blisters on the affected extremity, supporting the theory that CRPS1 is associated

with pathogenic local inflammation [10, 16]. This finding was confirmed in the current study. The involvement of TNF-α was further suggested by the success of anti-TNF treatment of CRPS1 [13] and the significant elevation of soluble TNF-α receptor type I (sTNF-RI) in CRPS1, particularly in combination with mechanical hyperalgesia [24]. Maihofner et al. (2005) reported that levels of this protein significantly correlate with TNF-α in CRPS1. Furthermore, elevated levels of IL-8 and sTNF-RI/II indicate an association between CRPS1 and an inflammatory process. Normal plasma levels of white blood cells, C-reactive protein, and IL-6 suggest a local process [25]. Another finding supporting the theory of an excessive inflammatory reaction is the increased extravasation of indium-labelled immunoglobulin as a sign of increased capillary permeability to macromolecules [26]. A recent finding suggests that there is an ongoing inflammatory response in the form of increased leukocyte accumulation on the involved side [27].

In contrast, several studies found no indication of an inflammatory process. No association was found between immune activation and CRPS1 [28], and no difference was found between patients and controls in the production of proinflammatory and anti-inflammatory cytokines in plasma [29]. The lack of high levels of inflammatory cytokines in the circulation supports the idea of local inflammation, as suggested by previous findings using the skin blister method [10, 23]. Patient selection might also contribute to the difference in findings. Both of the above studies included patients with longstanding CRPS1; it is possible that the immune response diminishes over the course of the disease.

Because levels of TNF-α and IL-6 were higher on the involved side in our group, one might expect that these markers of inflammation would be related to the characteristic signs and symptoms of inflammation. However, there was no correlation. An explanation for the absence of the correlation is that the changes in temperature, volume, AROM and pain in CRPS1 are irregularly distributed across the hand or foot.

In an ongoing inflammatory process, one would also expect a local increase in skin temperature and blood flow [1]. However, 41% of the patients in our study had a higher skin temperature for the involved extremity and 56% had a lower skin temperature for the involved extremity. There was no significant difference between these two groups in the levels of the proinflammatory cytokines.

Several studies confirm this observation, even describing a primarily cold CRPS1 [5, 11, 30, 31]. A possible explanation for this could be that blood flow, and thus skin temperature, is the result of not only afferent but also efferent mechanisms. Disturbances in central temperature regulation could result in altered (local) temperature of the injured extremity [32-34].

The profound changes in macrovascular and microvascular perfusion in patients with systemic inflammatory response syndrome (SIRS) and sepsis also contribute to edema in the CRPS1-affected arm and support an inflammatory-associated pathogenesis for some CRPS1 symptoms [25]. In this study swelling was reported by 96% of the patients, and in 87% swelling of the involved side was observed by a physician. Contrary to these findings, we measured edema on the involved side in only 39% of patients. No significant difference was found in the levels of TNF-α and IL-6 between the group with and without edema measured with the volumeter. It is possible that edema is also affected by other inflammatory mediators such as calcitonin gene-related peptide (CGRP) and substance P (SP), and related mechanisms. Alternatively, volumeter-based measurement of the differences between the involved and contralateral extremities might not be sufficiently accurate.

Earlier work revealed a slight decline in TNF-α and IL-6 between the acute stage (median 6 months, interquartile range 2-12 months) and the intermediate stage (median 30 months, interquartile range 23-40 months) of CRPS1, but showed that the levels are still significantly higher on the affected side. However, impairments of volume, mobility, and pain on the involved side, are significantly reduced compared to the acute stage. This suggests that the initiation and sustained development of CRPS1 are at least partially affected by proinflammatory cytokines. However, the mechanism is more complex, involving a number of cells, such as monocytes, tissue macrophages, and mast cells [16]. Another possible pathophysiologic mechanism for CRPS1 might be neurogenic inflammation, as suggested by the increased levels of systemic CGRP in patients with acute CRPS1 contributing to vasodilation, edema, and increased sweating. Improvement of the clinical signs and symptoms is followed by a significant reduction in CGRP levels [35]. Moreover, the increased release of neuropeptides in CRPS1 in response to transcutaneous electrical stimulation suggests facilitated neurogenic inflammation [36].

Other indications of neurogenic inflammation are the increased SP-induced plasma protein extravasation in CRPS1 patients in the affected and unaffected limbs [37] and the significant increase in IL-1β and IL-6, but not TNF-α, in the cerebrospinal fluid of CRPS1 patients versus controls [9].

In the first two years, a number of our patients exhibited reduced temperature and signs of atrophy. Disease duration did not correlate with cytokine levels. However, all patients fulfilled the criteria of Bruehl [14], suggesting that various subpopulations, as described by others, were included [30, 38, 39]. It would be interesting to investigate differences in immune parameters among these various subpopulations in future studies.

In conclusion, this study confirms the presence of local inflammation in CRPS1 in a large population of 66 patients in the first 2 years of the disease. Proinflammatory cytokines seem to be only partly involved in the pathophysiology, as indicated by the lack of coherence between levels of TNF-α and IL-6 and the signs and symptoms of inflammation and disease duration, as measured with the regularly used assessment instruments for CRPS1. Other inflammatory mediators and other mechanisms such as central sensitization are probably involved in the early stages of the disease.

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References

1. Janig, W. and R. Baron, *Complex regional pain syndrome: mystery explained?* Lancet Neurol, 2003. 2(11): p. 687-97.
2. de Mos, M., A.G. de Bruijn, F.J. Huygen, J.P. Dieleman, B.H. Stricker, and M.C. Sturkenboom, *The incidence of complex regional pain syndrome: a population-based study.* Pain, 2007. 129(1-2): p. 12-20.
3. Daemen, M., H. Kurvers, P. Bullens, G. Barendse, M. Van Kleef, and F. Van den Wildenberg, *Neurogenic inflammation and reflex sympathetic dystrophy (in vivo and in vitro assessment in an experimental model).* Acta Orthop Belg, 1998. 64(4): p. 441-7.
4. Daemen, M.A., H.A. Kurvers, P.J. Kitslaar, D.W. Slaaf, P.H. Bullens, and F.A. Van den Wildenberg, *Neurogenic inflammation in an animal model of neuropathic pain.* Neurol Res, 1998. 20(1): p. 41-5.
5. Veldman, P.H., H.M. Reynen, I.E. Arntz, and R.J. Goris, *Signs and symptoms of reflex sympathetic dystrophy: prospective study of 829 patients.* Lancet, 1993. 342(8878): p. 1012-6.
6. Birklein, F., B. Riedl, N. Sieweke, M. Weber, and B. Neundorfer, *Neurological findings in complex regional pain syndromes--analysis of 145 cases.* Acta Neurol Scand, 2000. 101(4): p. 262-9.
7. Birklein, F., D. Claus, B. Riedl, B. Neundorfer, and H.O. Handwerker, *Effects of cutaneous histamine application in patients with sympathetic reflex dystrophy.* Muscle Nerve, 1997. 20(11): p. 1389-95.
8. Huygen, F.J., A.G. de Bruijn, J. Klein, and F.J. Zijlstra, *Neuroimmune alterations in the complex regional pain syndrome.* Eur J Pharmacol, 2001. 429(1-3): p. 101-13.
9. Alexander, G.M., M.A. van Rijn, J.J. van Hilten, M.J. Perreault, and R.J. Schwartzman, *Changes in cerebrospinal fluid levels of pro-inflammatory cytokines in CRPS.* Pain, 2005. 116(3): p. 213-9.
10. Huygen, F.J., A.G. De Bruijn, M.T. De Bruin, J.G. Groeneweg, J. Klein, and F.J. Zijistra, *Evidence for local inflammation in complex regional pain syndrome type 1.* Mediators Inflamm, 2002. 11(1): p. 47-51.
11. Birklein, F., *Complex regional pain syndrome.* J Neurol, 2005. 252(2): p. 131-8.
12. Schwartzman, R.J. and A. Popescu, *Reflex sympathetic dystrophy.* Curr Rheumatol Rep, 2002. 4(2): p. 165-9.
13. Huygen, F.J., S. Niehof, F.J. Zijlstra, P.M. van Hagen, and P.L. van Daele, Successful treatment of CRPS 1 with anti-TNF. J Pain Symptom Manage, 2004. 27(2): p. 101-3.
14. Bruehl, S., R.N. Harden, B.S. Galer, S. Saltz, M. Bertram, M. Backonja, R. Gayles, N. Rudin, M.K. Bhugra, and M. Stanton-Hicks, *External validation of IASP diagnostic criteria for Complex Regional Pain Syndrome and proposed research diagnostic criteria.* International Association for the Study of Pain. Pain, 1999. 81(1-2): p. 147-54.
15. Huygen, F.J., N. Ramdhani, A. van Toorenenbergen, J. Klein, and F.J. Zijlstra, *Mast cells are involved in inflammatory reactions during Complex Regional Pain Syndrome type 1.* Immunol Lett, 2004. 91(2-3): p. 147-54.
16. Munnikes, R.J., C. Muis, M. Boersma, C. Heijmans-Antonissen, F.J. Zijlstra, and F.J. Huygen,

Intermediate stage complex regional pain syndrome type 1 is unrelated to proinflammatory cytokines. Mediators Inflamm, 2005(6): p. 366-72.

17. Carlsson, A.M., *Assessment of chronic pain. I. Aspects of the reliability and validity of the visual analogue scale.* Pain, 1983. 16(1): p. 87-101.
18. Hershler, C., T.A. Conine, A. Nunn, and M. Hannay, *Assessment of an infra-red non-contact sensor for routine skin temperature monitoring: a preliminary study.* J Med Eng Technol, 1992. 16(3): p. 117-22.
19. Fereidoni, M., A. Ahmadiani, S. Semnanian, and M. Javan, *An accurate and simple method for measurement of paw edema.* J Pharmacol Toxicol Methods, 2000. 43(1): p. 11-4.
20. Oerlemans, H.M., R.A. Oostendorp, T. de Boo, L. van der Laan, J.L. Severens, and J.A. Goris, *Adjuvant physical therapy versus occupational therapy in patients with reflex sympathetic dystrophy/complex regional pain syndrome type I. Arch Phys* Med Rehabil, 2000. 81(1): p. 49-56.
21. Perez, R.S., H.M. Oerlemans, W.W. Zuurmond, and J.J. De Lange, *Impairment level SumScore for lower extremity Complex Regional Pain Syndrome type I.* Disabil Rehabil, 2003. 25(17): p. 984-91.
22. Kemler, M.A., C.P. Rijks, and H.C. de Vet, *Which patients with chronic reflex sympathetic dystrophy are most likely to benefit from physical therapy?* J Manipulative Physiol Ther, 2001. 24(4): p. 272-8.
23. Heijmans-Antonissen, C., F. Wesseldijk, R.J. Munnikes, F.J. Huygen, P. van der Meijden, W.C. Hop, H. Hooijkaas, and F.J. Zijlstra,
    Multiplex bead array assay for detection of 25 soluble cytokines in blister fluid of patients with complex regional pain syndrome type 1. Mediators Inflamm, 2006(1): p. 28398.
24. Maihofner, C., H.O. Handwerker, B. Neundorfer, and F. Birklein, *Mechanical hyperalgesia in complex regional pain syndrome: a role for TNF-alpha?* Neurology, 2005. 65(2): p. 311-3.
25. Schinkel, C., A. Gaertner, J. Zaspel, S. Zedler, E. Faist, and M. Schuermann, *Inflammatory mediators are altered in the acute phase of posttraumatic complex regional pain syndrome.* Clin J Pain, 2006. 22(3): p. 235-9.
26. Oyen, W.J., I.E. Arntz, R.M. Claessens, J.W. Van der Meer, F.H. Corstens, and R.J. Goris, *Reflex sympathetic dystrophy of the hand: an excessive inflammatory response?* Pain, 1993. 55(2): p. 151-7.
27. Tan, E.C., W.J. Oyen, and R.J. Goris, *Leukocytes in Complex Regional Pain Syndrome type I.* Inflammation, 2005. 29(4-6): p. 182-6.
28. Ribbers, G.M., W.P. Oosterhuis, J. van Limbeek, and M. de Metz, *Reflex sympathetic dystrophy: is the immune system involved?* Arch Phys Med Rehabil, 1998. 79(12): p. 1549-52.
29. van de Beek, W.J., E.J. Remarque, R.G. Westendorp, and J.J. van Hilten, *Innate cytokine profile in patients with complex regional pain syndrome is normal.* Pain, 2001. 91(3): p. 259-61.
30. Vaneker, M., O.H. Wilder-Smith, P. Schrombges, I. de Man-Hermsen, and H.M. Oerlemans, *Patients initially diagnosed as 'warm' or 'cold' CRPS 1 show differences in central sensory processing some eight years after diagnosis: a quantitative sensory testing study.* Pain, 2005.

115(1-2): p. 204-11.

31. Vaneker, M., O.H. Wilder-Smith, P. Schrombges, and H.M. Oerlemans, *Impairments as measured by ISS do not greatly change between one and eight years after CRPS 1 diagnosis.* Eur J Pain, 2005. 18: p. 18.
32. Niehof, S.P., F.J. Huygen, R.W. van der Weerd, M. Westra, and F.J. Zijlstra, *Thermography imaging during static and controlled thermoregulation in complex regional pain syndrome type 1: diagnostic value and involvement of the central sympathetic system.* Biomed Eng Online, 2006. 5: p. 30.
33. Wasner, G., K. Heckmann, C. Maier, and R. Baron, *Vascular abnormalities in acute reflex sympathetic dystrophy (CRPS I): complete inhibition of sympathetic nerve activity with recovery.* Arch Neurol, 1999. 56(5): p. 613-20.
34. Wasner, G., J. Schattschneider, K. Heckmann, C. Maier, and R. Baron, *Vascular abnormalities in reflex sympathetic dystrophy (CRPS I): mechanisms and diagnostic value.* Brain, 2001. 124(Pt 3): p. 587-99.
35. Birklein, F., M. Schmelz, S. Schifter, and M. Weber, *The important role of neuropeptides in complex regional pain syndrome.* Neurology, 2001. 57(12): p. 2179-84.
36. Weber, M., F. Birklein, B. Neundorfer, and M. Schmelz, *Facilitated neurogenic inflammation in complex regional pain syndrome.* Pain, 2001. 91(3): p. 251-7.
37. Leis, S., M. Weber, A. Isselmann, M. Schmelz, and F. Birklein, *Substance-P-induced protein extravasation is bilaterally increased in complex regional pain syndrome.* Exp Neurol, 2003. 183(1): p. 197-204.
38. Harden, R.N. and S.P. Bruehl, *Diagnosis of complex regional pain syndrome: signs, symptoms, and new empirically derived diagnostic criteria.* Clin J Pain, 2006. 22(5): p. 415-9.
39. Bruehl, S., R.N. Harden, B.S. Galer, S. Saltz, M. Backonja, and M. Stanton-Hicks, *Complex regional pain syndrome: are there distinct subtypes and sequential stages of the syndrome?* Pain, 2002. 95(1-2): p. 119-24.

Chapter 3

Six years follow-up of the levels of TNF-α and IL-6 in patients with CRPS type 1

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Abstract
In an earlier study, levels of the proinflammatory cytokines TNF-α and IL-6 are higher in blisters fluid from the CRPS1 (complex regional pain syndrome type 1) side obtained at 6 and 30 months (median) after the initial event. The aim of this follow-up study is to determine the involvement of these cytokines in long lasting CRPS1. Twelve CRPS1 patients, with median disease duration of 72 months, participated. The levels of TNF-α and IL-6 were measured in blister fluid; disease activity was reevaluated by measuring pain and differences in temperature, volume, and mobility between both extremities. Differences in levels of IL-6 and TNF-α and mobility between both sides were significantly decreased. Pain and differences in temperature and volume were not significantly altered. No correlation was found between the cytokines and the disease characteristics. These results indicate that IL-6 and TNF-α are only partially responsible for the signs and symptoms of CRPS1.

Introduction
Complex regional pain syndrome type 1 (CRPS1) is a disease of an extremity that usually occurs as a complication after surgery or trauma, although spontaneous occurrence is also described [1].
The pathophysiology of CRPS1 is still not totally clear. In general, three mechanisms are thought to be involved: afferent mechanisms (e.g. neurogenic inflammation) [2-4], efferent mechanisms (e.g. autonomic disturbances) [5], and central nervous system mechanisms (e.g. cerebral plasticity) [6]. Based on our review of the literature regarding the CRPS pathophysiology, we hypothesized that following a trauma or surgery, the normal sterile inflammatory response runs out of control, and is perhaps initiated by a genetic and/or acquired immunologic disorder [7]. Neuroimmune activation of cells in the peripheral nervous system, which is part of the afferent mechanism, apparently results in central sensitization and exacerbation of pain [8]. Neuropeptides, cytokines, and other mediators are released during the inflammation [9, 10] and cause the prominent signs and symptoms, which resemble inflammation; these include increased skin temperature, edema, pain, loss of function, and redness [7, 11].
Levels of TNF-α and IL-6 were previously shown to be elevated in fluid of artificially induced skin blisters from the CRPS1 side in the initial stage of the disease [9, 12, 13]. These observations

suggest local inflammation. We hypothesized that in most patients local inflammation would only be present during the first year of the disease, since the clinical signs and symptoms of CRPS1 are expected to diminish over time in most patients. Therefore, we predicted that the formation of proinflammatory mediators (such as IL-6 and TNF-α) should decline during the course of the disease. In an earlier study, we showed that although the levels of TNF-α and IL-6 declined in the intermediate stage of the disease, they were still significantly elevated in the CRPS1 extremity [13]. In the present study, we examine whether this decline in cytokine levels continues during the course of the disease and whether this decline is correlated with a possible improvement in disease activity as measured by registration of pain, and by the measurement of differences in temperature, volume, and mobility between the CRPS1 and contralateral extremities.

Materials and methods

The protocol was approved by the Medical Ethics Committee of the Erasmus MC Rotterdam (MEC no. 1989.780/2001/24). Guidelines according to the Declaration of Helsinki (amended version of 2002) and Good Clinical Practice (ICH/GCP version 1996) were followed. Data collection and calculations were performed according to guidelines for registration of personal data.

Patients

Sixty-six patients with CRPS1 in one extremity for 7 months after the initial event participated in several studies conducted between April 2001 and February 2004 (T0) to investigate the pathophysiology of CRPS1 or the effects of specific treatments for CRPS1 [9, 12, 14]. At the time of the first follow-up study in 2004, 25 patients with CRPS1 with a median disease duration of 30 months after the initial event agreed to participate (T1) [13]. In 2007 these 25 patients were again asked to participate. One patient had died, one patient appeared to have CRPS type 2, and 11 patients chose not to participate again. In the end, 12 patients with CRPS1 were included in this study (T2).

For this study, we used the results obtained at baseline (T0), first follow-up measurement (T1) and second follow-up measurement (T2) from only these 12 patients. All 12 patients fulfilled the CRPS1 criteria by Bruehl et al. [15] at the first measurement (T0), performed shortly after the initial event (median 4 months) which resulted in the development of the disease.

Pain assessment
The intensity of pain was assessed by using a visual analogue scale (VAS) recorded in 0–100 millimeters [16]. The McGill Pain Questionnaire, Dutch Language Version (MPQ-DLV), was measured by counting the total number of words chosen from 20 items [17].

Temperature measurement
Skin temperature was measured using an infrared tympanic probe thermometer (First Temp Genius®; Sherwood Medical Crawley, Sussex, UK) [18]. Measurements were obtained on the dorsal aspect of the hand in a matrix of five points. The difference in mean temperature between the CRPS1 and contralateral extremities was calculated, and the data were expressed with respect to the temperature of the unaffected hand.

Assessment of volume
Volume was measured with a volumeter, which measures the amount of water displaced by immersion of a body part [19]. The difference between the CRPS1 and contralateral extremities was calculated as a percentage of the contralateral extremity.

Assessment of mobility
Mobility was assessed by measuring the active range of motion (AROM), which is defined as the arc of motion requiring muscle power to achieve the motion of a joint [20, 21]. The AROM on the CRPS1 extremity was multiplied by 100 and divided by the AROM on the contralateral extremity to derive the percentage of normal mobility.

Blisters
Artificial skin blisters were induced using a suction method [9, 14, 22]. A skin suction chamber was positioned on the skin of the CRPS1 and contralateral extremities. A vacuum of 300 mm Hg was applied with an Atmoforte 350A aspirator pump (ATMOS Medizintechnik, Lenzkirch, Germany). After 15 min, the vacuum was reduced to 250 mm Hg, and after another 15 min it was reduced to 200 mm Hg. This negative pressure was maintained for 2-2.5 h. The blisters created were punctured, and fluid was pooled from each side into a 1.5-ml Eppendorf conical polypropylene tube and centrifuged for 5 min at 1600 x g. All samples

were stored in 1-ml conical polypropylene tubes at -80 °C until analysis [9, 22].

Cytokine assays

Blister fluid samples were diluted 4-fold in appropriate calibrator diluent assay buffer for the direct measurement of cytokines. Cytokine assays were performed following the manufacturer's protocol (PeliKine™ human ELISA kits for IL-6 [M1906] and TNF-α [M1920]; CLB, Amsterdam, the Netherlands). The standard curve ranges and mean calculated zero signal ± 3 (SD) were 0-80 and 0.3 pg/ml for IL-6 and 0-1000 and 1 pg/ml for TNF-α. The absorbance per well was measured at 450 nm with a Medgenix EASIA reader. Sample concentrations were calculated using the appropriate standard calibration lines and the Softmax® software of the reader.

Statistical analysis

Data were analyzed with SPSS ® for Windows, version 14.0. To determine whether the 12 CRPS1 patients from this study were a representative group of the initial 66 CRPS1 patients who started the follow-up study, the one-way ANOVA test was used for a comparison between groups for the pro-inflammatory cytokines, and the outcome parameters pain, temperature, volume and mobility. To determine if the differences in means for these selected parameters were significantly different from 0, we applied the one-sample t-test for comparisons of the levels of the pro-inflammatory cytokines between both extremities, and to the differences in the outcome parameters of temperature, volume, and mobility. Mixed model analysis was used to compare the differences between both extremities to each other for the levels of the cytokines, VAS, McGill, temperature, volume and mobility between each time point. Correlations between the differences in levels of IL-6 and TNF-α and the outcome parameters were calculated using the Pearson Correlation test. Significance was accepted at the $p < 0.05$ (two-sided) level.

Results

The group of 12 CRPS1 patients was a good representation of the original group of 66 CRPS1 patients with which we initiated the follow-up study 6 years ago. The groups were equal for difference in levels of IL-6 ($p=0.60$) and TNF-α ($p=0.72$). They were also

equal for the McGill pain score (p=0.41), temperature difference (p=0.41), volume difference (p=0.48), and mobility (p=0.83). The two groups were only significantly different from each other for VAS pain (p=0.03). The characteristics and medication use of the 12 patients with CRPS1 in one extremity for 6 years (median) who were examined 3 times during the follow-up of their CRPS1 are presented in Table 1.

Table 1. Characteristics of the study population

Patient characteristic		Value		
Gender:	male/female	3/9		
Side:	right/left	6/6		
Cause:	fracture/accident/surgery/spontaneous	6/2/4/0		
		T0	T1	T2
Age in years		52 (48-56)	54 (51-58)	57 (54-62)
Duration of CRPS1 in months		4 (3-14)	35 (21-48)	72 (59-86)
Disease related medication (number of patients)				
	Non-steroid anti-inflammatory drugs (NSAIDs)	6	1	0
	Opiates	4	2	2
	Anti-oxidants	8	1	0
	Vasodilators	1	0	0
	Muscle relaxants	1	0	0
	Anti-depressants	1	1	0
	Benzodiazepines	2	0	0
	Anti-epileticum	1	0	1

Data are presented as the n or median (interquartile range).

Table 2. Levels of IL-6 and TNF-α in blister fluid obtained from the CRPS1 and contralateral extremities at first measurement (T0), second measurement (T1) and third measurement (T2). Data are presented as mean (range).

	T0		T1		T2	
	CRPS1	Contralateral	CRPS1	Contralateral	CRPS1	Contralateral
IL-6	116*	8	80*	2	22	20
(pg/ml)	(5-662)	(1-36)	(1-346)	(0-5)	(4-78)	(3-61)
TNF-α	66*	31	56*	16	38	47
(pg/ml)	(1-359)	(1-258)	(3-176)	(2-80)	(9-81)	(10-142)

* Wilcoxon signed-ranks test $p<0.05$ (CRPS1 vs contralateral).

The IL-6 and TNF-α levels in blister fluid at all time intervals are presented in Table 2. The differences in levels of IL-6 between both extremities did not significantly change at T1 compared to T0 ($p=0.531$) and at T2 compared to T0 ($p=0.063$). However, the differences in levels did significantly decrease at T2 compared to T1 ($p=0.028$). The differences in levels of TNF-α between both extremities did not significantly change at T1 compared to T0 ($p=0.892$) and at T2 compared to T0 ($p=0.153$). The decrease in differences from T1 to T2 almost reached significance ($p=0.064$). At the T0 and T1 measurement, the difference in levels of IL-6 and TNF-α between the CRPS1 side and the contralateral side in blister fluid were significantly different from 0. However, at the T2 measurement, no significant differences between the two sides were evident (Figure 1a and 1b) for the 12 CRPS1 patients.
Also the signs and symptoms of impairment were measured at T0, T1 and T2 in terms of pain and differences in temperature, volume, and mobility between the CRPS1 and the contralateral extremities. The VAS pain did not significantly change during the course of the disease in these 12 patients ($p=0.472$). The McGill pain score improved significantly at T1 compared to T0 ($p=0.006$), but pain was significantly worse at T2 compared to T1 ($p=0.041$). Furthermore, no significant improvement was seen at T2 as compared to T0 ($p=0.567$). The VAS pain and the McGill pain score significantly differed from 0 during all 6 years of follow-up (Fig. 2a and 2b).

Figure 1a.

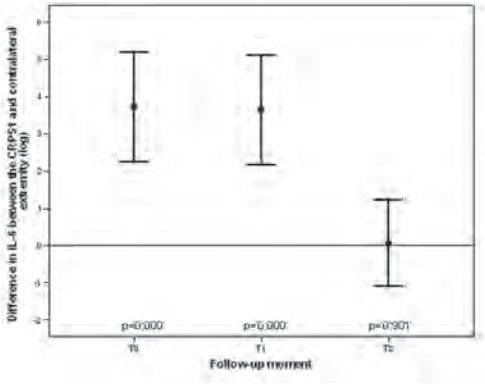

Figure 1b.

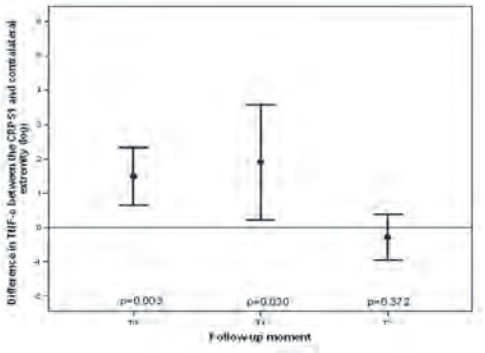

Figure 1. Differences in levels of IL-6 and in TNF-α in blisterfluid between the CRPS1 and contralateral extremities over the course of CRPS1. Blister fluid was collected as described in Materials and Methods and IL-6 (panel a) and TNF-α (panel b) levels (pg/ml) in the CRPS1 and the contralateral limbs were measured by ELISA. The data obtained from the same 12 patients at each time period are expressed as the difference in IL-6 or TNF-α levels between the two sides (log pg/ml). Each time point shows the mean α the standard deviation. The p values represent the deviation from no difference (0).

Figure 2a.

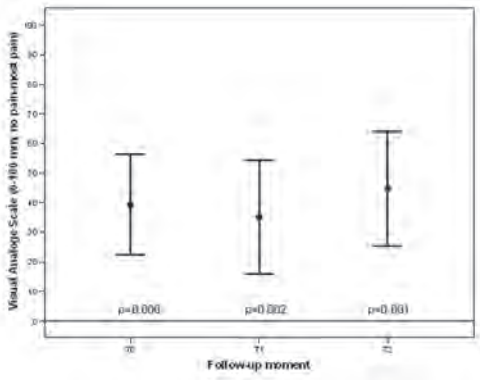

Figure 2b.

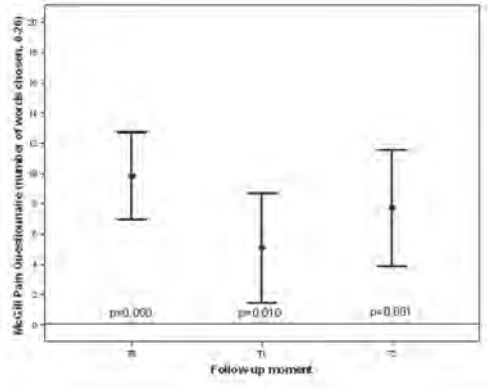

Figure 2c.

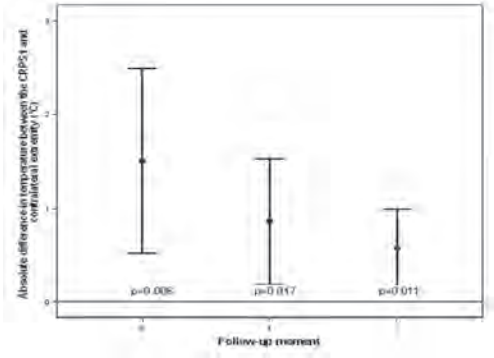

Figure 2d.

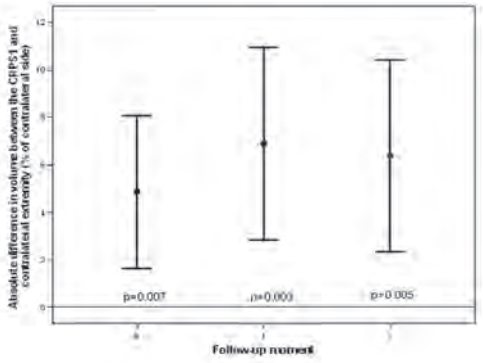

Figure 2e.

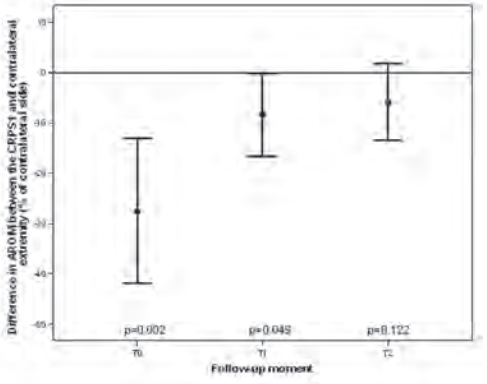

Figure 2. The assessment of pain over the course of CRPS1 disease as measured by the Visual Analogue Scale is shown in panel (a). Panel (b) shows the assessment of pain as measured by the McGill Pain Questionnaire. Panel (c), (d) and (e) respectively show the absolute differences in skin surface temperature, the absolute differences in volume, and the differences in AROM between the CRPS1 and contralateral sides in CRPS1 patients over the course of disease. The data obtained from the same 12 patients at the 3 points of measurement were collected as described in Materials and Methods and are expressed as the mean ± standard deviation. The p values represent the deviation from no difference (0).

The absolute difference in temperature and volume between both sides did not change during the course of the disease (p=0.204 and p=0.509 respectively). The difference between the CRPS1 and contralateral side did vary significantly from 0 at all three moments of measurement for temperature and volume (Fig. 2c). Mobility improved significantly at T1 and T2 as compared to T0 (p=0.002 for both), with no significant increase in AROM at T2 compared to T1 (p=0.353). The difference in mobility between both sides was significantly different from 0 at T0 and T1, but no significant difference was found at T2 (Fig. 2e).
No correlation was found between the differences in levels of the proinflammatory cytokines and the other disease-related parameters pain and differences between both extremities for temperature, volume and mobility during the course of the disease.
The total use of disease related medication was divided in 8 categories (see Table 1). The total number of patients using medication as well as the variety in types of medication decreased during the course of the disease.

Discussion

In a six years follow-up study in 12 CRPS1 patients, we found a decrease in the extent of the differences in levels of TNF-α and IL-6 in blister fluid. After disease duration of 4 months (T0) and of 3 years (T1), the levels of these cytokines were significantly higher in the CRPS1 extremity compared to the contralateral extremity. However, after 6 years (T2) the differences in the cytokine levels between the two extremities were not significantly different. In contrast, the difference in mobility between both sides was significantly improved after 6 years. Pain and the differences in temperature and volume were not significantly altered during the course of the disease. No correlation was found between the pro-inflammatory cytokines and the disease characteristics.
Because TNF-α and IL-6 are proinflammatory cytokines, one might expect that these markers of inflammation would be directly related to the characteristics of inflammation: pain, temperature increase, edema, and loss of function. In this study the levels of TNF-α and IL-6 were diminished during the course of the disease, but no clear improvement of inflammatory signs was found. Most of the 12 patients still reported much pain. The VAS score did not change during the course of the disease, and

while the McGill Pain score improved at first, it worsened again at the third measurement. The absolute temperature difference tended to diminish during the course of the disease; however, this was not significant. The volume difference also did not change significantly during the course of the disease. Only the AROM improved significantly after 6 years; the patients only described some stiffness of the joints.

No improvement of the signs and symptoms of inflammation was found as described above, however after six years, a decline in the number of patients using medication that could counteract inflammation has been observed, such as NSAIDs, opiates and antioxidants. We concluded that the normalization of inflammatory mediators after six years was not affected through pharmacological intervention, but due to a diminution of disease activity.

An explanation for a decrease in differences in the levels of TNF-α and IL-6 between both sides during the course of CRPS1 is a spreading of the disease from the CRPS1 side to the contralateral extremity. This spreading could also be explained by the fact that the levels in the CRPS1 side are increased during the course of the disease. However, the increased levels of the contralateral side measured 6 years after the initial event do not even come close to the high levels of TNF-α and IL-6 measured in the CRPS1 side 4 months and 3 years after the initial event. Furthermore, the levels of IL-6 of both sides at T2 are within the normal range of the levels measured in blister fluid from healthy controls (mean 16 pg/ml) [23]. These findings do not support the theory of spreading. Finally, one would expect that the differences between both sides in disease activity would decrease during the course of the disease when there is spreading. This is not confirmed in this follow-up study.

In the present study, in contrast with the diminished levels of the proinflammatory cytokines during the course of the disease, signs of inflammation were found in all three stages of the disease. In an earlier study, during the first two years of CRPS1, about half of the patients showed no inflammatory signs [24], but did fulfil the criteria of Bruehl [15]. Several other studies confirmed that observation, even describing a primarily cold CRPS1 [4, 10, 25, 26].

An explanation for finding a temperature increase and decrease already at the onset of CRPS1, is that blood flow, and thus changes in skin temperature, are the result of not only afferent

mechanisms, such as local mediators, but also efferent mechanisms. Disturbances in central temperature regulation could result in altered (local) temperature of the injured extremity [27-29]. Recently we found evidence that temperature changes also might be partly caused by the crosstalk in the vascular system between higher levels of cytokines and the nitric oxide/endothelin-1 (NO/ET-1) balance [30, 31]. Furthermore, blood flow and tissue-blood distribution could be diminished, partly due to disuse of the extremity [13].

A possible explanation for finding edema and atrophy during all stages of the disease is that edema is not only affected by TNF-α and IL-6 but also by other inflammatory mediators such as calcitonin gene-related peptide (CGRP) and substance P (SP), and related mechanisms [32, 33].

No correlation was found between the differences in levels of the cytokines and pain and differences between both extremities for temperature, volume and AROM for all three times of measurement. In an earlier study, we found similar results [24]. Perhaps IL-6 and TNF-α are only partially responsible for the permanent damage in CRPS1, expressed as pain, and changes in temperature, volume, and mobility. Other mediators or a combination of mediators, such as nitric oxide [30] and/or amino acids [34, 35] or other mechanisms [7] may also play a role in the pathophysiology of CRPS1 and may explain in part the course of the disease.

Acknowledgement

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Reference

1. Janig, W. and R. Baron, Complex regional pain syndrome: mystery explained? Lancet Neurol, 2003. 2(11): p. 687-97.
2. Daemen, M., H. Kurvers, P. Bullens, G. Barendse, M. Van Kleef, and F. Van den Wildenberg, Neurogenic inflammation and reflex sympathetic dystrophy (in vivo and in vitro assessment in an experimental model). Acta Orthop Belg, 1998. 64(4): p. 441-7.
3. Daemen, M.A., H.A. Kurvers, P.J. Kitslaar, D.W. Slaaf, P.H. Bullens, and F.A. Van den Wildenberg, Neurogenic inflammation in an animal model of neuropathic pain. Neurol Res, 1998. 20(1): p. 41-5.
4. Veldman, P.H., H.M. Reynen, I.E. Arntz, and R.J. Goris, Signs and symptoms of reflex sympathetic dystrophy: prospective study of 829 patients. Lancet, 1993. 342(8878): p. 1012-6.
5. Birklein, F., B. Riedl, N. Sieweke, M. Weber, and B. Neundorfer, Neurological findings in complex regional pain syndromes--analysis of 145 cases. Acta Neurol Scand, 2000. 101(4): p. 262-9.
6. Birklein, F., D. Claus, B. Riedl, B. Neundorfer, and H.O. Handwerker, Effects of cutaneous histamine application in patients with sympathetic reflex dystrophy. Muscle Nerve, 1997. 20(11): p. 1389-95.
7. Huygen, F.J., A.G. de Bruijn, J. Klein, and F.J. Zijlstra, Neuroimmune alterations in the complex regional pain syndrome. Eur J Pharmacol, 2001. 429(1-3): p. 101-13.
8. Alexander, G.M., M.A. van Rijn, J.J. van Hilten, M.J. Perreault, and R.J. Schwartzman, Changes in cerebrospinal fluid levels of pro-inflammatory cytokines in CRPS. Pain, 2005. 116(3): p. 213-9.
9. Huygen, F.J., A.G. De Bruijn, M.T. De Bruin, J.G. Groeneweg, J. Klein, and F.J. Zijistra, Evidence for local inflammation in complex regional pain syndrome type 1. Mediators Inflamm, 2002. 11(1): p. 47-51.
10. Birklein, F., Complex regional pain syndrome. J Neurol, 2005. 252(2): p. 131-8.
11. Schwartzman, R.J. and A. Popescu, Reflex sympathetic dystrophy. Curr Rheumatol Rep, 2002. 4(2): p. 165-9.
12. Huygen, F.J., S. Niehof, F.J. Zijlstra, P.M. van Hagen, and P.L. van Daele, Successful treatment of CRPS 1 with anti-TNF. J Pain Symptom Manage, 2004. 27(2): p. 101-3.
13. Munnikes, R.J., C. Muis, M. Boersma, C. Heijmans-Antonissen, F.J. Zijlstra, and F.J. Huygen, Intermediate stage complex regional pain syndrome type 1 is unrelated to proinflammatory cytokines. Mediators Inflamm, 2005(6): p. 366-72.
14. Huygen, F.J., N. Ramdhani, A. van Toorenenbergen, J. Klein, and F.J. Zijlstra, Mast cells are involved in inflammatory reactions during Complex Regional Pain Syndrome type 1. Immunol Lett, 2004. 91(2-3): p. 147-54.
15. Bruehl, S., R.N. Harden, B.S. Galer, S. Saltz, M. Bertram, M. Backonja, R. Gayles, N. Rudin, M.K. Bhugra, and M. Stanton-Hicks, External validation of IASP diagnostic criteria for Complex Regional Pain Syndrome and proposed research diagnostic criteria. International Association for the Study of Pain. Pain, 1999. 81(1-2): p. 147-54.
16. Carlsson, A.M., Assessment of chronic pain. I. Aspects of the reliability and validity of the visual analogue scale. Pain, 1983. 16(1): p. 87-101.

17. Lowe, N.K., S.N. Walker, and R.C. MacCallum, Confirming the theoretical structure of the McGill Pain Questionnaire in acute clinical pain. Pain, 1991. 46(1): p. 53-60.
18. Hershler, C., T.A. Conine, A. Nunn, and M. Hannay, Assessment of an infra-red non-contact sensor for routine skin temperature monitoring: a preliminary study. J Med Eng Technol, 1992. 16(3): p. 117-22.
19. Fereidoni, M., A. Ahmadiani, S. Semnanian, and M. Javan, An accurate and simple method for measurement of paw edema. J Pharmacol Toxicol Methods, 2000. 43(1): p. 11-4.
20. Oerlemans, H.M., R.A. Oostendorp, T. de Boo, L. van der Laan, J.L. Severens, and J.A. Goris, Adjuvant physical therapy versus occupational therapy in patients with reflex sympathetic dystrophy/complex regional pain syndrome type I. Arch Phys Med Rehabil, 2000. 81(1): p. 49-56.
21. Kemler, M.A., C.P. Rijks, and H.C. de Vet, Which patients with chronic reflex sympathetic dystrophy are most likely to benefit from physical therapy? J Manipulative Physiol Ther, 2001. 24(4): p. 272-8.
22. Heijmans-Antonissen, C., F. Wesseldijk, R.J. Munnikes, F.J. Huygen, P. van der Meijden, W.C. Hop, H. Hooijkaas, and F.J. Zijlstra, Multiplex bead array assay for detection of 25 soluble cytokines in blister fluid of patients with complex regional pain syndrome type 1. Mediators Inflamm, 2006(1): p. 28398.
23. Schmidt, E., B. Bastian, R. Dummer, H.P. Tony, E.B. Brocker, and D. Zillikens, Detection of elevated levels of IL-4, IL-6, and IL-10 in blister fluid of bullous pemphigoid. Arch Dermatol Res, 1996. 288(7): p. 353-7.
24. Wesseldijk, F., F.J. Huygen, C. Heijmans-Antonissen, S.P. Niehof, and F.J. Zijlstra, Tumor necrosis factor-alpha and interleukin-6 are not correlated with the characteristics of Complex Regional Pain Syndrome type 1 in 66 patients. Eur J Pain, 2008. 12(6): p. 716-21.
25. Vaneker, M., O.H. Wilder-Smith, P. Schrombges, I. de Man-Hermsen, and H.M. Oerlemans, Patients initially diagnosed as 'warm' or 'cold' CRPS 1 show differences in central sensory processing some eight years after diagnosis: a quantitative sensory testing study. Pain, 2005. 115(1-2): p. 204-11.
26. Vaneker, M., O.H. Wilder-Smith, P. Schrombges, and H.M. Oerlemans, Impairments as measured by ISS do not greatly change between one and eight years after CRPS 1 diagnosis. Eur J Pain, 2005. 18: p. 18.
27. Wasner, G., K. Heckmann, C. Maier, and R. Baron, Vascular abnormalities in acute reflex sympathetic dystrophy (CRPS I): complete inhibition of sympathetic nerve activity with recovery. Arch Neurol, 1999. 56(5): p. 613-20.
28. Wasner, G., J. Schattschneider, K. Heckmann, C. Maier, and R. Baron, Vascular abnormalities in reflex sympathetic dystrophy (CRPS I): mechanisms and diagnostic value. Brain, 2001. 124(Pt 3): p. 587-99.
29. Niehof, S.P., F.J. Huygen, R.W. van der Weerd, M. Westra, and F.J. Zijlstra, Thermography imaging during static and controlled thermoregulation in complex regional pain syndrome type 1: diagnostic value and involvement of the central sympathetic system. Biomed Eng Online, 2006. 5: p. 30.

30. Groeneweg, J.G., F.J. Huygen, C. Heijmans-Antonissen, S. Niehof, and F.J. Zijlstra, Increased endothelin-1 and diminished nitric oxide levels in blister fluids of patients with intermediate cold type complex regional pain syndrome type 1. BMC Musculoskelet Disord, 2006. 7: p. 91.
31. Huygen, F.J., S. Niehof, J. Klein, and F.J. Zijlstra, Computer-assisted skin videothermography is a highly sensitive quality tool in the diagnosis and monitoring of complex regional pain syndrome type I. Eur J Appl Physiol, 2004. 91(5-6): p. 516-24.
32. Gradl, G., B. Finke, S. Schattner, P. Gierer, T. Mittlmeier, and B. Vollmar, Continuous intra-arterial application of substance P induces signs and symptoms of experimental complex regional pain syndrome (CRPS) such as edema, inflammation and mechanical pain but no thermal pain. Neuroscience, 2007. 148(3): p. 757-65.
33. Leis, S., M. Weber, M. Schmelz, and F. Birklein, Facilitated neurogenic inflammation in unaffected limbs of patients with complex regional pain syndrome. Neurosci Lett, 2004. 359(3): p. 163-6.
34. Wesseldijk, F., D. Fekkes, F.J. Huygen, M. van de Heide-Mulder, and F.J. Zijlstra, Increased plasma glutamate, glycine, and arginine levels in complex regional pain syndrome type 1. Acta Anaesthesiol Scand, 2008. 52(5): p. 688-94.
35. Wesseldijk, F., D. Fekkes, F.J. Huygen, E. Bogaerts-Taal, and F.J. Zijlstra, Increased plasma serotonin in complex regional pain syndrome type 1. Anesth Analg, 2008. 106(6): p. 1862-7.

Chapter 4

Increased plasma glutamate, glycine, and arginine levels in CRPS type 1

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Abstract
Various inflammatory mediators have been identified as potential contributors to complex regional pain syndrome type 1 (CRPS1), but these mediators do not entirely explain certain manifestations of the syndrome, such as pain. The objective of this study was to investigate the role of amino acids in the pathogenesis of CRPS1. We used HPLC to determine plasma concentrations of 16 amino acids, especially those related to the NMDA receptor (e.g., glutamate and glycine) and nitric oxide synthesis (e.g., arginine and citrulline) in patients with CRPS1 (n = 64) and age- and sex-matched healthy controls (n = 51). Patients rated pain intensity (visual analog scale) and the subjective experience of pain intensity (McGill Pain Questionnaire). Psychological dysfunction was assessed using the SCL-90. Relative to controls, in CRPS1 patients' plasma levels of glutamate, arginine, taurine and glycine were increased, and plasma levels of glutamine and the ratio of citrulline to arginine were decreased. Remarkably, in CRPS1 patients there was a highly significant inverse correlation between glutamine and glutamate, although the sum of molar concentrations of glutamate and glutamine remains unchanged. Subjective measures of pain and indicators of psychoneuroticism and emotional instability did not correlate with amino acid levels. This study shows for the first time a pronounced increase in amino acid levels in this chronic pain syndrome. The marked differences in glutamate, glutamine, glycine, taurine and arginine levels between patients and controls suggest the involvement of both the NDMA receptor and the endothelium-dependent arginine-nitric oxide system in CRPS1.

Introduction
Complex regional pain syndrome type 1 (CRPS1) is a disease of severe chronic pain in an extremity; it usually develops after trauma or surgery. In addition to pain, CRPS1 is characterized by other sensory and autonomic, motor, and dystrophic signs and symptoms [1]. These include changes in skin surface temperature, edema, hypersensitivity, and allodynia. In the chronic phase of CRPS1, patients report continuous pain and temperature change in the affected extremity, an indication of perturbed blood tissue distribution. All signs are indicative of local neurogenic inflammation [2, 3]. In most cases, CRPS1 leads to a dramatic loss of function and disability. The subsequent phases and related mecha-

nisms underlying the disease are poorly understood. Therefore the availability of effective treatment is still missing.

Inflammation

Some of the prominent signs and symptoms of CRPS1 (e.g., redness, increased skin temperature and edema) could be explained by formation of neuropeptides [2] or proinflammatory cytokines [4, 5]. CRPS1 may involve a process of central sensitization, which exacerbates pain, through neuroimmune activation of cells in the peripheral nervous system [6]. In general, standard pharmacological and interventional therapy successively provide insufficient suppression of the spread of CRPS1 from the distal part to the entire extremity [3]. Therefore, it is likely that other mechanisms, which result in the release of potent pain-related substances, are involved. In open trials, some patients with CRPS1 responded well to the N-methyl-D-aspartate (NMDA) receptor antagonist ketamine [7-10], providing indirect evidence for the involvement of specific amino acids (such as glutamate and glycine) in CRPS1. Also, activation of monocytes, macrophages, and mast cells after injury could lead to a pronounced increase in proinflammatory cytokines and chemokines in CRPS1 [4, 5].

Ischemia

Permanent damage to nerve endings can reduce both endothelium-dependent dilatation and regional blood distribution [11]. Disturbance of the microcirculation causes ischemia, which elicits pain and inhibits oxidative phosphorylation. By virtue of the cellular structure of the microvessels, endothelial and smooth muscle cells are the first to confront the metabolic change. Recently, we demonstrated an imbalance between endothelium-derived endothelin-1 and nitric oxide (NO) in CRPS1 [12]. This could be due to activation of endothelial cells or smooth muscle cells by proinflammatory cytokines or to changes in levels of arginine.

Central effects

Continuous inflammation could result in nociceptor sensitization and/or increased release of substances modulating neurons and glia [6]. Recently it was found that glutamate is released into the cerebrospinal fluid of CRPS1 patients [13]. However, to our knowledge, plasma amino acid levels in CRSP1 have not previously been assessed.

Based on the literature we hypothesize that in CRPS1 patients the amino acids which bind to the pain-related NMDA receptor (e.g., glutamate and glycine) would be increased, and that, due to endothelial dysfunction, the NO-related synthesis of the amino acids arginine and citrulline could be affected.
The aim of this study was therefore to investigate plasma concentrations of a number of amino acids in order to determine whether changes in the plasma levels of the hypothesized amino acids display some specificity for CRPS1. We compared absolute levels of amino acids between patients with CRPS1 and age- and sex-matched healthy controls, and assessed correlations with pain visual analog scores, McGill pain intensity, and quality of life factors.

Methods

The protocol was approved by the Medical Ethics Committee of the Erasmus Medical Center, Rotterdam (MEC no. 1989.780/2001/24). All procedures were performed in accordance with the Helsinki declaration. Written informed consent was obtained from all patients.

Subjects

All CRPS1 patients were referred from the southwest Netherlands to the pain clinic of the Erasmus Medical Center. Sixty-four patients diagnosed with CRPS1 in one extremity were recruited. Most of the patients were in the early chronic phase of the disease, with a mean disease duration of 34.7 months. Diagnosis of the patients was established based on the medical history and the anamnesis to record the signs of the disease and based on physical examination to record the CRPS1-specific symptoms according to the criteria developed by Bruehl et al. [14]. Fifty-one age- and sex-matched healthy controls recruited from the employers of the Erasmus Medical Center, living in southwest Netherlands, also participated. Participant demographic characteristics are presented in Table 1.

Blood samples and amino acid determinations

EDTA blood samples (7 ml) were collected by venipuncture from patients and healthy controls. In order to detect local synthesis of amino acids, in each patient blood samples were obtained from the CRPS1 affected extremity and a contralateral extremity. For

Table 1 Demographic data and characteristics of the patients and healthy controls

	Value
Number of patients	64
Males/females	15/49
Number of healthy controls	51
Males/females	11/40
Mean age (years)	
Patients	48 ± 1.6
Healthy controls	48 ± 1.3
Localization of CRPS1 at diagnosis	
Upper extremity	50
Lower extremity	14
Mean time between onset of the disease and plasma sample taken for amino acids measurement (months)	34.7 ± 5.1
Disease-related medication (number of patients used)	
Non-steroid anti-inflammatory drugs (NSAIDs)	29
Opiates	21
Anti-oxidants	25
Vasodilators	20
Muscle relaxants	6
Anti-depressants	10
Benzodiazepines	6

Means ± SEM; healthy controls were age and sex matched in comparison with CRPS1 patients

the 50 patients with CRPS1 in the upper extremity, blood was obtained from the cubital vein of the CRPS1extremity and the contralateral upper extremity. For the 14 patients with CRPS1 in the lower extremity, blood was obtained from the femoral vein of the CRPS1 extremity and the cubital vein of the contralateral upper extremity. For the healthy controls, one blood sample was obtained from the cubital vein of an upper extremity.

Plasma was isolated by centrifugation at 2650 gmax for 10 min at 20 °C, and samples were stored at -80 °C until assay. Plasma was deproteinized with 5-sulphosalicylic acid (6%, w/v) containing norvaline and homoserine as internal standards. Amino acids were assayed by high-performance liquid chromatography (HPLC) using automated precolumn derivatization with o-phthaldialdehyde and fluorescence detection [15]. The following amino

acids were determined: glutamate, glutamine, arginine, citrulline, serine, glycine, taurine, methionine, lysine, ornithine, isoleucine, leucine, valine, phenylalanine, tyrosine and tryptophan. The limit of detection depends on the amino acid in question, because of different fluorescence responses and differing peak shapes of the deriv¬atives. Typi¬cal values were 54 fmol for glutamate and 167 fmol for serine. Concentrations of amino acids down to 0.5 μmol/L in plasma can be measured accurately with our method. The interassay coefficient of variation was for all amino acids below 4%.

Visual analogue scale, McGill Pain Questionnaire, and SCL-90

During patients' visit to the pain clinic, pain intensity was determined using a visual analogue scale (VAS) [16] and the McGill Pain Questionnaire Dutch-Language Version (MPQ-DLV) (17). The VAS is measured in millimeters (0, no pain; 100, most intense pain). The MPQ-DLV score is calculated by counting the total number of words chosen (0-20) [17].

Patients completed the Dutch language version of the SCL-90 [18], a multidimensional, self-report inventory comprising 90 items measuring dimensions such as anxiety, agoraphobia, depression, somatization, inadequacy, sensitivity, hostility, and insomnia. The total score is a measure of psychoneuroticism and emotional instability.

Statistical analysis

Data were analyzed with SPSS for Windows, version 12.0.1. Variables are reported as the mean ± SEM. Group differences were assessed using the t-test for independent samples; we used the Bonferroni correction for multiple comparisons, resulting in an alpha-level of 0.003. Differences in plasma amino acid concentrations between CRPS1 and contralateral extremities were evaluated using the Wilcoxon paired samples test. Protection for multiple Correlations between age, sex, disease duration, pain, quality of life and amino acids were examined with Pearson's correlation test. P-values were two-sided, and a p-value < 0.05 was considered statistically significant.

Results

At diagnosis, CRPS1 was mostly localized to the upper extremity

(Table 1). In 13 patients, it was inappropriate to obtain blood from the affected extremity; therefore, blood was obtained from both the CRPS1 extremity and the contralateral side in 51 patients. For all amino acids measured, paired samples of the CRPS1 extremity and the contralateral extremity were highly correlated ($r > 0.90$ and $p < 0.001$). An example is shown in Fig. 1.

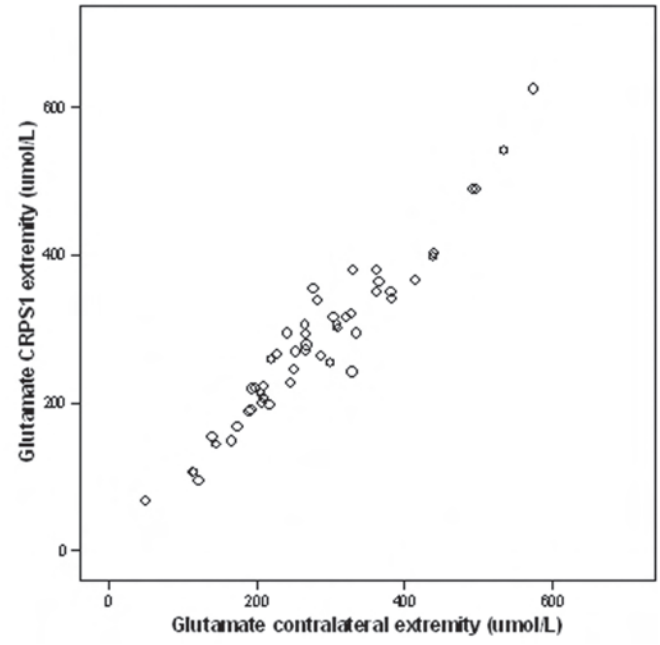

Figure 1. Representative example of paired sample analysis of plasma amino acid concentrations (μmol/L). Plasma glutamate concentrations in samples obtained from the CRPS1 extremity and the contralateral extremity are shown ($r = 0.96$; $p < 0.001$).

Based on these results, for further analysis and comparison to healthy controls, of whom only one sample was obtained from one upper extremity, for each amino acid the individual mean plasma value of the affected side and contralateral side was determined. Table 2 shows the mean values of 16 amino acids in plasma of all patients and those of the healthy controls. For a number of amino acids, the mean concentration was significantly different between patients and healthy controls. However, in terms of absolute change in μmol/L amino acid, the remarkable differences were for glutamate, glutamine, arginine, taurine, serine, and glycine (Fig. 2).

Table 2 Amino acid concentrations in plasma of CRPS1 patients and healthy controls

Amino acid	CRPS1 patients (n=64)		Healthy controls (n=51)	
	mean	SEM	mean	SEM
Glutamate	270*	14.5	47	2.8
Glutamine	330*	14.9	559	11.9
Arginine	171*	5.2	71	3.1
Citrulline	33	1.4	35	1.2
Serine	160*	3.0	111	2.8
Glycine	267*	7.0	216	5.9
Taurine	95*	4.9	42	1.1
Methionine	15*	1.2	31	0.9
Lysine	203	5.0	185	5.0
Ornithine	88	3.3	86	3.2
Isoleucine	83	3.0	82	3.8
Leucine	157	5.3	147	4.4
Valine	260	7.4	285	8.2
Phenylalanine	78*	1.8	62	1.4
Tyrosine	68	2.4	70	2.2
Tryptophan	51	1.2	50	1.6

Concentrations in μmol/L. * $P<0.003$

We investigated correlations between these six amino acids and found that there was a highly significant inverse correlation between glutamate and glutamine for patients (Fig. 3; $r = - 0.79$, $p < 0.001$), but not for healthy controls.

Subgroup analysis was performed for disease-related medication (non-steroid anti-inflammatory drugs (NSAIDs), opiates, anti-oxidants, vasodilators, muscle relaxants, anti-depressants and benzodiazepines) and amino acids. No correlations were observed.

A weak but significant correlation was found between duration of CRPS1 (in months) and plasma arginine levels ($r = 0.41$; $p = 0.001$). Furthermore, a slight but significant difference in plasma arginine levels was found between CRPS1 in the upper and lower extremity (178 ± 5.3 μmol/L and 148 ± 13 μmol/L, respectively; $p = 0.015$). The ratio of citrulline to arginine was significantly lower in CRPS1 patients versus healthy controls (0.203 ± 0.011 versus 0.519 ± 0.023; $p < 0.001$).

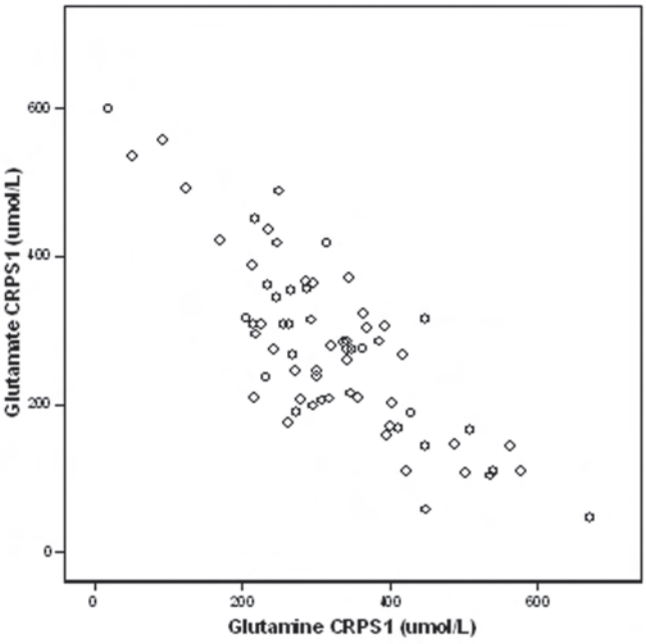

Figure 2. Mean absolute change in plasma amino acids in patients relative to healthy controls.

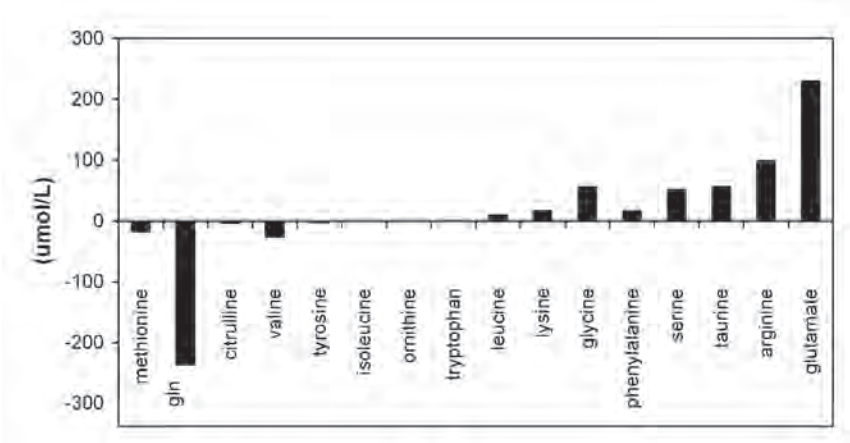

Figure 3. Correlation between plasma levels of glutamate and glutamine. Levels were highly correlated in CRPS1 patients (upper panel; $r = -0.79$, $p < 0.001$), but they were not correlated in healthy controls (lower panel). gln: glutamine.

The mean intensity of pain was moderate: the mean VAS score in CRPS1 patients was 48 ± 3.4 mm, and the McGill pain intensity rating was 11.7 ± 0.59. VAS and McGill questionnaire scores did not correlate with amino acid levels. Thus, as an example, there was no correlation between VAS scores and glutamate levels ($r = -0.03$ and $p = 0.823$) and McGill questionnaire and glutamate levels ($r = 0.10$ and $p = 0.470$).
The mean SCL-90 score was 140 ± 6.2, which is comparable to the score in a normal population, and in the same range as in previously studied CRPS1 patients [19]. The mean score did not correlate with amino acid levels. There was a slight but significant correlation between the SCL-90 score and VAS score ($r = 0.37$ and $p = 0.004$) and between the SCL-90 score and the McGill pain intensity rating ($r = 0.34$ and $p = 0.02$).

Discussion

The main findings of this study are a pronounced increase in plasma levels of glutamate, arginine, glycine and taurine and a pronounced decrease in plasma glutamine in CRPS1 patients compared with age- and sex-matched healthy controls. No differences were observed between plasma samples drawn from the CRPS1 extremity and the contralateral extremity; paired samples were highly correlated. This indicates that changes in amino acid levels are not necessarily evoked at the site affected by CRPS1.
We also observed a highly significant inverse correlation between glutamine and glutamate. The sum of molar concentrations of glutamate and glutamine remains unchanged suggesting altered interconversion of glutamate and glutamine. Glutamine is converted into glutamate by the enzyme glutaminase. Furthermore, it is known that glutamine is used for the synthesis of urea in the liver, for renal ammoniagenesis and for gluconeogenesis in both liver and kidney and as a major respiratory fuel for many cells [20]. In burn patients, however, in whom immunological function and wound healing are the most prominent mechanisms, only reduced levels of plasma glutamine have been found [21]. Although it is generally accepted that glutamate will not pass the blood-brain barrier, plasma concentrations of glutamate are correlated with those in cerebrospinal fluid of ischemic stroke patients [22].
Surprisingly, amino acid levels did not correlate with subjective parameters of pain and indicators of psychoneuroticism or emotional instability.

Some amino acids are released in chronic, painful diseases, such as migraine. Apparently there are some similarities between the underlying mechanisms of CRPS1 and patients with migraine. Alam et al. [23] found that plasma glutamate, glutamine, and glycine levels were all elevated in patients with migraine compared to healthy controls (a mean increase of approximately 200, 150 and 280 μmol/L respectively; ranges comparable with our data), whereas there were no such differences for patients with tension headache [23]. In a recent study, plasma glutamate levels were found to be increased in migraine patients, particularly patients affected by migraine with aura, compared to healthy controls [24]. In a study of patients with chronic migraine, with and without fibromyalgia (assigned as pressure allodynia), glutamate levels were higher in patients with versus without fibromyalgia [25].

Plasma levels of glutamate, glutamine, glycine, and taurine were reported to be significantly increased in patients with depression compared to controls (a mean increase of approximately 35, 160, 105 and 85 μmol/L respectively), and a positive correlation was found between glutamate levels and the rate of depression [26]. Psychological abnormalities have been described in CRPS patients [27]; both the chronicity of the disease and the continuous pain contribute to these phenomena. However, patients in the current study did not exhibit specific psychological dysfunction. There was no direct correlation between subsets of the SCL-90 and amino acid levels. This was also true for subjective measures of pain, pain VAS scores and McGill pain intensity.

Glycine is a co-agonist of the NMDA receptor and known to modulate immune cell responses in which the inhibitory effects on macrophages could be important [28]. As noted earlier, some open trials suggest that the NMDA receptor antagonist ketamine suppresses neuropathic pain, which mainly manifests as allodynia and hyperalgesia [29]. Multi-day low-dose ketamine infusion for treatment of CRPS1 acts through antagonism of the NMDA receptor, resulting in a significant reduction of pain intensity and an improvement of mobility [8]. Also, ketamine reduces the capsaicin-evoked intensity and unpleasantness of mechanical hyperalgesia [30].

The increase in plasma arginine was not accompanied by a change in citrulline levels. In addition, the concentration of ornithine was not different between CPRS1 patients and controls. These observations point to reduced activity of one or more of

the NO synthase isoenzymes rather than changes in the activity of ornithine carbamoyltransferase, argininosuccinate synthase, or arginase. The higher plasma arginine levels in the patients may be partly due to reduced glutamine levels, because glutamine inhibits the generation of arginine in cultured endothelial cells [31]. Plasma arginine levels correlated with the duration of CRPS1 (in months), an indication that conversion of arginine to citrulline and NO is altered during the course of the disease. This could result in vasoconstriction, diminished tissue blood distribution, and spread of pain [12].

The citrulline/arginine ratio, regarded as an index of NO synthesis [32], was decreased in our CRPS1 patients. Although this decrease was due to a marked increase in plasma arginine, it is nevertheless probable that NO synthesis was decreased rather than increased, resulting in vasoconstriction of blood vessels in the CRPS1 extremity. During the early chronic episode of the disease, most of our patients exhibited a cold extremity, indicative of vasoconstriction [33]. This could also be due to increased levels of taurine, which were two-fold higher relative to controls. The release of taurine in various cell-damaging conditions, including ischemia and increased free radical production, have been shown [34]. Taurine in turn has antioxidative properties [35]. Very recently it has been shown that taurine could diminish neuropathic nociception, possibly through interaction with the glycine receptor [36]. It has been reported that taurine reduces cyclic GMP, which evokes vasodilation [37]. This suggests that application of a phosphodiesterase inhibitor would restore the cGMP-induced vasodilation and therefore could be beneficial in CRPS1. Another possibility is supplementation with glutamine, which not only was significantly reduced in our patients, but may also be beneficial for increasing NO synthase activity, theoretically resulting in greater conversion of arginine to NO [38]. However, only 1% of the total arginine flux is used for NO synthesis; therefore a change in the formation of the latter substance is very unlikely to affect arginine levels dramatically [39].

Analgesics and other medication used by the patients were not correlated with changes in the amino acid profile. Some limitations of this study are the lack of information concerning nutritional behaviour of CRPS1 patients in comparison with healthy controls. Furthermore, we failed to register body weight and length (resulting in the BMI) of all subjects. Nevertheless, in our

opinion the marked changes found in the CRPS1 group not only could be affected by these parameters.
In conclusion, our descriptive study shows for the first time a pronounced increase in amino acid levels in this chronic pain syndrome. The marked differences in glutamate, glutamine, glycine, taurine and arginine levels between patients and healthy controls suggest the involvement of both the endothelium-dependent arginine-nitric oxide system and the NDMA receptor in CRPS1. No proof, however, could be provided that the changes in plasma amino acids are causally related to the pathogenesis of CRPS1. After all, no correlations with measures of pain and indicators of psycho neuroticism and emotional instability were found. A randomized controlled trial, in which the effects of ketamine administration on both disease activity in CRPS1 patients and amino acids is being studied, should confirm the involvement of specific amino acids in this disease.

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References

1. Janig, W. and R. Baron, Is CRPS I a neuropathic pain syndrome? Pain, 2006. 120(3): p. 227-9.
2. Birklein, F., M. Schmelz, S. Schifter, and M. Weber, The important role of neuropeptides in complex regional pain syndrome. Neurology, 2001. 57(12): p. 2179-84.
3. Huygen, F.J., A.G. de Bruijn, J. Klein, and F.J. Zijlstra, Neuroimmune alterations in the complex regional pain syndrome. Eur J Pharmacol, 2001. 429(1-3): p. 101-13.
4. Huygen, F.J., A.G. De Bruijn, M.T. De Bruin, J.G. Groeneweg, J. Klein, and F.J. Zijistra, Evidence for local inflammation in complex regional pain syndrome type 1. Mediators Inflamm, 2002. 11(1): p. 47-51.
5. Heijmans-Antonissen, C., F. Wesseldijk, R.J. Munnikes, F.J. Huygen, P. van der Meijden, W.C. Hop, H. Hooijkaas, and F.J. Zijlstra, Multiplex bead array assay for detection of 25 soluble cytokines in blister fluid of patients with complex regional pain syndrome type 1. Mediators Inflamm, 2006(1): p. 28398.
6. Birklein, F., Complex regional pain syndrome. J Neurol, 2005. 252(2): p. 131-8.
7. Correll, G.E., J. Maleki, E.J. Gracely, J.J. Muir, and R.E. Harbut, Subanesthetic ketamine infusion therapy: a retrospective analysis of a novel therapeutic approach to complex regional pain syndrome. Pain Med, 2004. 5(3): p. 263-75.
8. Goldberg, M.E., R. Domsky, D. Scaringe, R. Hirsh, J. Dotson, I. Sharaf, M.C. Torjman, and R.J. Schwartzman, Multi-day low dose ketamine infusion for the treatment of complex regional pain syndrome. Pain Physician, 2005. 8(2): p. 175-9.
9. Villanueva-Perez, V.L., G. Cerda-Olmedo, J.M. Samper, A. Minguez, V. Monsalve, M.J. Bayona, and J.A. De Andres, Oral ketamine for the treatment of type I complex regional pain syndrome. Pain Pract, 2007. 7(1): p. 39-43.
10. Koffler, S.P., B.M. Hampstead, F. Irani, J. Tinker, R.T. Kiefer, P. Rohr, and R.J. Schwartzman, The neurocognitive effects of 5 day anesthetic ketamine for the treatment of refractory complex regional pain syndrome. Arch Clin Neuropsychol, 2007. 22(6): p. 719-29.
11. Schattschneider, J., K. Hartung, M. Stengel, J. Ludwig, A. Binder, G. Wasner, and R. Baron, Endothelial dysfunction in cold type complex regional pain syndrome. Neurology, 2006. 67(4): p. 673-5.
12. Groeneweg, J.G., F.J. Huygen, C. Heijmans-Antonissen, S. Niehof, and F.J. Zijlstra, Increased endothelin-1 and diminished nitric oxide levels in blister fluids of patients with intermediate cold type complex regional pain syndrome type 1. BMC Musculoskelet Disord, 2006. 7: p. 91.
13. Alexander, G.M., M.J. Perreault, E.R. Reichenberger, and R.J. Schwartzman, Changes in immune and glial markers in the CSF of patients with Complex Regional Pain Syndrome. Brain Behav Immun, 2007. 21(5): p. 668-76.
14. Bruehl, S., R.N. Harden, B.S. Galer, S. Saltz, M. Bertram, M. Backonja, R. Gayles, N. Rudin, M.K. Bhugra, and M. Stanton-Hicks, External validation of IASP diagnostic criteria for Complex Regional Pain Syndrome and proposed research diagnostic criteria. International Association for the Study of Pain. Pain, 1999. 81(1-2): p. 147-54.
15. Fekkes, D., A. van Dalen, M. Edelman, and A. Voskuilen, Validation of the determination

of amino acids in plasma by high-performance liquid chromatography using automated pre-column derivatization with o-phthaldialdehyde. J Chromatogr B Biomed Appl, 1995. 669(2): p. 177-86.
16. Merskey, H. and N. Bogduk, Classification of chronic pain: descriptions of chronic pain syndromes and definitions of pain terms. 1994, IASP Press: Seattle, WA. p. 40-43.
17. Lowe, N.K., S.N. Walker, and R.C. MacCallum, Confirming the theoretical structure of the McGill Pain Questionnaire in acute clinical pain. Pain, 1991. 46(1): p. 53-60.
18. Arrindell, W.A. and H. Ettema, The Dutch version of the Symptom Checklist (SCL-90). Ned Tijdsch Psych (Dutch J Psychol), 1981. 36: p. 77-108.
19. Geertzen, J.H., A.T. de Bruijn-Kofman, H.P. de Bruijn, H.B. van de Wiel, and P.U. Dijkstra, Stressful life events and psychological dysfunction in Complex Regional Pain Syndrome type I. Clin J Pain, 1998. 14(2): p. 143-7.
20. Curthoys, N.P. and M. Watford, Regulation of glutaminase activity and glutamine metabolism. Annu Rev Nutr, 1995. 15: p. 133-59.
21. Peng, X., H. Yan, Z. You, P. Wang, and S. Wang, Glutamine granule-supplemented enteral nutrition maintains immunological function in severely burned patients. Burns, 2006. 32(5): p. 589-93.
22. Castillo, J., F. Martinez, E. Corredera, J.M. Aldrey, and M. Noya, Amino acid transmitters in patients with headache during the acute phase of cerebrovascular ischemic disease. Stroke, 1995. 26(11): p. 2035-9.
23. Alam, Z., N. Coombes, R.H. Waring, A.C. Williams, and G.B. Steventon, Plasma levels of neuroexcitatory amino acids in patients with migraine or tension headache. J Neurol Sci, 1998. 156(1): p. 102-6.
24. Vaccaro, M., C. Riva, L. Tremolizzo, M. Longoni, A. Aliprandi, E. Agostoni, A. Rigamonti, M. Leone, G. Bussone, and C. Ferrarese, Platelet glutamate uptake and release in migraine with and without aura. Cephalalgia, 2007. 27(1): p. 35-40.
25. Peres, M.F., E. Zukerman, C.A. Senne Soares, E.O. Alonso, B.F. Santos, and M.H. Faulhaber, Cerebrospinal fluid glutamate levels in chronic migraine. Cephalalgia, 2004. 24(9): p. 735-9.
26. Mitani, H., Y. Shirayama, T. Yamada, K. Maeda, C.R. Ashby, Jr., and R. Kawahara, Correlation between plasma levels of glutamate, alanine and serine with severity of depression. Prog Neuropsychopharmacol Biol Psychiatry, 2006. 30(6): p. 1155-8.
27. Rommel, O., A. Willweber-Strumpf, P. Wagner, D. Surall, J.P. Malin, and M. Zenz, [Psychological abnormalities in patients with complex regional pain syndrome (CRPS)]. Schmerz, 2005. 19(4): p. 272-84.
28. Schilling, T. and C. Eder, A novel physiological mechanism of glycine-induced immunomodulation: Na+-coupled amino acid transporter currents in cultured brain macrophages. J Physiol, 2004. 559(Pt 1): p. 35-40.
29. Jorum, E., T. Warncke, and A. Stubhaug, Cold allodynia and hyperalgesia in neuropathic pain: the effect of N-methyl-D-aspartate (NMDA) receptor antagonist ketamine--a double-blind, cross-over comparison with alfentanil and placebo. Pain, 2003. 101(3): p. 229-35.
30. Poyhia, R. and A. Vainio, Topically administered ketamine reduces capsaicin-evoked

mechanical hyperalgesia. Clin J Pain, 2006. 22(1): p. 32-6.
31. Sessa, W.C., M. Hecker, J.A. Mitchell, and J.R. Vane, The metabolism of L-arginine and its significance for the biosynthesis of endothelium-derived relaxing factor: L-glutamine inhibits the generation of L-arginine by cultured endothelial cells. Proc Natl Acad Sci U S A, 1990. 87(21): p. 8607-11.
32. Fekkes, D., M. Bannink, W.H. Kruit, A.R. Van Gool, P.G. Mulder, S. Sleijfer, A.M. Eggermont, and G. Stoter, Influence of pegylated interferon-alpha therapy on plasma levels of citrulline and arginine in melanoma patients. Amino Acids, 2007. 32(1): p. 121-6.
33. Wasner, G., J. Schattschneider, K. Heckmann, C. Maier, and R. Baron, Vascular abnormalities in reflex sympathetic dystrophy (CRPS I): mechanisms and diagnostic value. Brain, 2001. 124(Pt 3): p. 587-99.
34. Saransaari, P. and S.S. Oja, Characteristics of taurine release induced by free radicals in mouse hippocampal slices. Amino Acids, 2004. 26(1): p. 91-8.
35. Zhang, M., I. Izumi, S. Kagamimori, S. Sokejima, T. Yamagami, Z. Liu, and B. Qi, Role of taurine supplementation to prevent exercise-induced oxidative stress in healthy young men. Amino Acids, 2004. 26(2): p. 203-7.
36. Pellicer, F., A. Lopez-Avila, U. Coffeen, J. Manuel Ortega-Legaspi, and R.D. Angel, Taurine in the anterior cingulate cortex diminishes neuropathic nociception: a possible interaction with the glycine(A) receptor. Eur J Pain, 2007. 11(4): p. 444-51.
37. Hilgier, W., E. Anderzhanova, S.S. Oja, P. Saransaari, and J. Albrecht, Taurine reduces ammonia- and N-methyl-D-aspartate-induced accumulation of cyclic GMP and hydroxyl radicals in microdialysates of the rat striatum. Eur J Pharmacol, 2003. 468(1): p. 21-5.
38. Bellows, C.F. and B.M. Jaffe, Glutamine is essential for nitric oxide synthesis by murine macrophages. J Surg Res, 1999. 86(2): p. 213-9.
39. Luiking, Y.C. and N.E. Deutz, Isotopic investigation of nitric oxide metabolism in disease. Curr Opin Clin Nutr Metab Care, 2003. 6(1): p. 103-8.

Chapter 5

Increased plasma serotonin in CRPS type 1

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Abstract
In patients with complex regional pain syndrome type 1 (CRPS1), some improvement can be achieved by the administration of ketanserin, a 5-HT2A receptor antagonist. Our aim was to measure plasma levels of serotonin (5-HT) during CRPS1 and correlate these levels with disease characteristics. Plasma 5-HT was measured in 35 patients with CRPS1 for 3 years and compared with those of 35 age-matched healthy controls. The plasma 5-HT levels were 411 ± 263 nmol/l and 29 ± 18 nmol/l, respectively ($P < 0.001$). No correlations with disease characteristics were observed. The markedly elevated levels of plasma 5-HT in CRPS1 patients suggest a role for 5-HT during the course of this disease. However, because of the lack of correlations with distinct disease characteristics, 5-HT is probably one of a number of mediators in CRPS1.

Introduction
Complex regional pain syndrome type 1 (CRPS1) is a complication that follows trauma or surgery in an extremity, leading to a dramatic loss of function, disability, and chronic pain. Therapeutic options are limited and insufficient to provoke complete recovery [1-4]. In an animal model, prolonged disturbance of the microcirculation by ischemia/reperfusion causes pain and other signs of CRPS1, such as oedema and changes in skin temperature [5]. Similar signs have been observed after cardiac catheterization via the radial artery [6, 7].
In blood vessels, serotonin (5-hydroxy-tryptamine; 5-HT) evokes vasoconstriction through activation of the 5-HT2A receptor. Important actions and functions of 5-HT are: i) vascular constriction and contraction of other smooth muscles; ii) increased micro vascular permeability; iii) induction of blood platelet aggregation; iv) stimulation of peripheral nociceptive nerve endings and v) excitation/inhibition of neurons in the central nervous system. Clinical conditions associated with disturbed 5-HT function include migraine, carcinoid syndrome, mood disorders, and anxiety [8, 9].
Specific 5-HT2A receptor antagonism by ketanserin results in a pronounced vasodilatation, which was demonstrated in CRPS1 patients two decades ago. Two case reports indicated that ketanserin was an effective vasodilator in sympathetic dystrophy or causalgia (currently known as CRPS1) [10, 11], and a double-

blind placebo-controlled crossover trial of 16 patients with chronic peripheral burning pain demonstrated the beneficial effect of ketanserin [12]. Based on that report, ketanserin is also recommended for clinical use in chronic CRPS1 [13]. However, no studies investigating 5-HT levels in the CRPS1 affected limb or in the circulation that would justify this type of pharmaceutical intervention have been performed [14].
Our aim was to investigate whether CRPS1 patients had detectable increases in plasma 5-HT concentrations during the course of the disease. Some patients with CRPS1 exhibit besides the described physical limitations also psychological dysfunction [15], and this phenomenon could be associated with increased 5-HT [16]. The co-occurrence of increased 5-HT with chronic pain suggests that 5-HT could be a common causative factor in chronic pain and psychological dysfunction [17]. Therefore, we also compared plasma 5-HT levels with pain, disease activity (skin surface temperature, oedema and mobility) and a self-report inventory.

Methods

The protocol was approved by the Medical Ethics Committee of the Erasmus MC Rotterdam (MEC no. 1989.780/2001/24) and written informed patient consent was obtained. Guidelines according to the Declaration of Helsinki (amended version of 2002) and Good Clinical Practice (ICH/GCP version 1996) were followed. Data collection and calculations were performed according to guidelines for registration of personal data.

Subjects

A total of 35 patients diagnosed as having CRPS1 in one extremity according to the criteria of Bruehl [18] were recruited for this study.
A group of 35 age-matched healthy controls were asked to give blood for plasma 5-HT analysis. Demographic data and characteristics of the patients and controls are presented in Table 1, including any disease-related medication. None of the patients used ketanserin.

Blood samples

EDTA blood samples (7 ml) were collected from the patients and healthy controls. In 29 patients with CRPS1 in the upper extremity, blood was obtained from both the CRPS1 and the contralateral

Table 1 Demographic data and characteristics of the patients and healthy controls

Number of patients	35
Males/females	9/26
Number of healthy controls	35
Males/females	20/15
Mean age (years)	
Patients	48 ± 12
Healthy controls	48 ± 6.1
Localization of CRPS1 at diagnosis	
Upper extremity	29
Lower extremity	6
Mean time between onset of the disease and plasma sample taken for serotonin measurement (months)	36 ± 41
Disease-related medication (number of patients used)	
Non-steroid anti-inflammatory drugs (NSAIDs)	12
Opiates	4
Anti-oxidants	13
Vasodilators	3
Muscle relaxants	1
Anti-depressants	2
Benzodiazepines	4

Means ± SD; healthy controls were age matched with CRPS1 patients

extremity by venapuncture. In 6 patients with CRPS1 in the lower extremity, blood was drawn from the CRPS1 extremity, and a control sample taken from the contralateral upper extremity by venapuncture. For the healthy controls, one sample of blood was drawn from an upper extremity by venapuncture. Blood was centrifuged at 2650×gmax for 10 min at 20°C and plasma was stored at –80°C until assay.

Plasma serotonin determinations

Measurement of plasma 5-HT concentration, and its break-down product 5-hydroxyindoleacetic acid (5-HIAA), was performed using reversed-phase HPLC method according to Bax et al. [19]. The detection limit for both compounds was approximately 3 fmol at a signal to noise ratio of 3. The mean recoveries (± SD) of 5-HT and 5-HIAA added to the platelet poor plasma (PPP) samples were 95 ± 7% (n = 76) and 72 ± 8% (n = 35), respectively.

Pain measurements

The maximal intensity of disease-related pain during the last 24 hrs was determined using a visual analogue scale (VAS) [20, 21] and the McGill pain questionnaire Dutch-Language Version (MPQ-DLV) [22]. The VAS is measured in millimetres (0-100, no pain to most intensive pain), and the MPQ-DLV score is measured by counting the total number of words chosen (0-20).

Measurements of disease activity

1. Skin temperature measurements

Skin surface temperature also reflects blood tissue distribution. It is generally accepted that vasoconstriction or vasodilatation respectively leads to cold or warm extremities [1].

We measured skin temperature on the dorsal aspect of the hand or foot in a matrix of five points using an infrared tympanic probe thermometer (First Temp Genius®; Sherwood Medical Crawley, Sussex, UK) [23], and calculated the difference in mean temperature between the CRPS1 and the contralateral extremity.

2. Determination of oedema

We measured differences in the volume (in g) of the CRPS1 and contralateral extremities using a volumeter, which measures the amount of water displaced by the immersion of a body part [24].

3. Determination of mobility

We assessed mobility in the upper and lower extremity, by measuring the active range of motion (AROM) according to the American Society of Hand Therapists clinical assessment recommendations [25-27]. The AROM on the CRPS1 extremity was multiplied by 100 and divided by the AROM on the contralateral extremity, resulting in percentage of normal mobility (100%).

Self-report inventory

Patients completed the Dutch-Language Version of the SCL-90 [28]. The SCL-90 is a multidimensional, self-report inventory composed of 90 items that measures dimensions such as anxiety, agoraphobia, depression, somatization, inadequacy, sensitivity, hostility, and insomnia. The total score (between 90 and 450) is a measure of psychoneuroticism or emotional instability.

Statistical analysis

Data were analyzed with SPSS for Windows, version 12.0. The one-sample Kolmogorov-Smirnov test was used to determine whether or not the sample data were consistent with the normal distribution function. Data of temperature difference, volume difference, AROM, VAS pain, McGill pain, SCL-90, and the plasma 5-HT concentration were reported as mean ± SD. Between subjects, differences in plasma 5-HT concentrations between extremities were analyzed using the Wilcoxon signed-ranks test, and the correlation between parameters was calculated using the Pearson's correlation coefficient. Differences in plasma 5-HT between patients and controls were tested by the Mann-Whitney test. The alpha of the tests was set at the traditional 0.05 level.

Results

5-HT

Plasma 5-HT levels in CRPS1 affected extremities (491 ± 280 nmol/l) and the contralateral extremities (433 ± 260 nmol/l) were in the same range and correlated well with one other ($r = 0.97$; $P < 0.001$). However, we observed a marked difference between the plasma 5-HT levels of healthy controls and patients (Table 2), both in CRPS1-hands (441 ± 250 nmol/l; n = 29) and CRPS1 feet (265 ± 300 nmol/l; n = 6) in comparison with healthy controls. No differences were observed between males (390 ± 255 nmol/l; n = 9) and females (418 ± 270 nmol/l; n = 26). Plasma 5-HT levels were not correlated with disease duration (correlation coefficient 0.152, P-value 0.384). 5-HIAA was slightly and significantly decreased in CRPS1 patients (Table 2). Again, no differences were observed between males (26 ± 15 nmol/l; n = 6) and females (25 ± 10 nmol/l; n = 17).

Table 2. Plasma 5-HT and 5-HIAA concentrations in CRPS1 patients and healthy controls

	CRPS1 patients (n = 35)		Healthy controls (n = 35)		
	mean	SD	mean	SD	P-value
5-HT (nmol/l)	411	263	29	18	< 0.001
5-HIAA (nmol/l)	26	11	41	9	< 0.001

Pain

The mean VAS pain in this patient group (n=35) was 54 ± 28 mm on a scale of 0-100 mm, whereas the McGill pain intensity (measured in n=26) was 12 ± 5 words out of 20 possible chosen words. Neither VAS pain nor McGill pain intensity correlated with plasma 5-HT (Table 3). Figure 1A and 1B show the distribution of plasma 5-HT concentrations plotted against VAS pain and McGill pain intensity.

Objective disease activity parameters and plasma 5-HT correlations

Functional disease parameters have been measured effectively in a proportion of the patients. The temperature difference (n=29), the volume difference (n=22) and the AROM (n=22) did not correlate with plasma 5-HT levels (Table 3 and Figures 1C, 1D and 1E respectively).

Self-report inventory

The total score of the SCL-90 (n=35) as a measure of psycho neuroticism or emotional instability in this group of patients was 145 ± 50, which is not significantly increased in comparison with earlier published total scores from a normal population, and is in the same range as the CRPS1 patients from that study [15]. Sub-scale scores and the total score did not correlate with plasma 5-HT levels (Table 3 and Figure 1F).

Table 3. Correlations between plasma 5-HT and other outcome measures

plasma 5-HT and:	Correlation coefficient	P-value
VAS pain	- 0.14	0.429
McGill pain questionnaire	- 0.12	0.547
temperature difference	- 0.28	0.145
volume difference	0.39	0.073
mobility (AROM)	- 0.38	0.088
SCL-90 questionnaire	- 0.32	0.079

Figure 1A

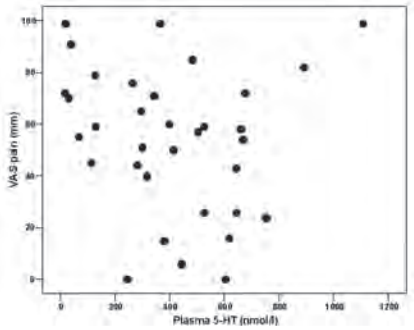

Figure 1B

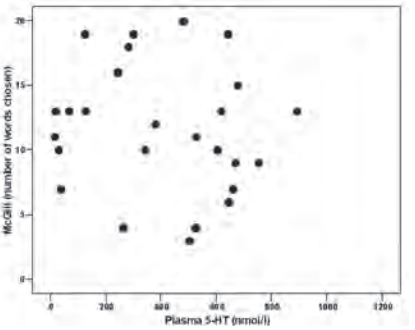

Figure 1C

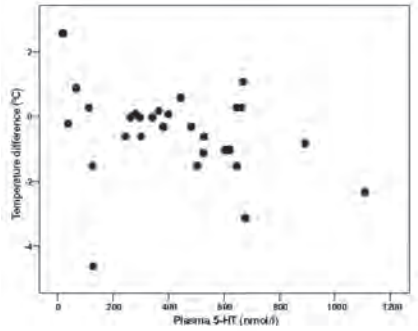

Figure 1D

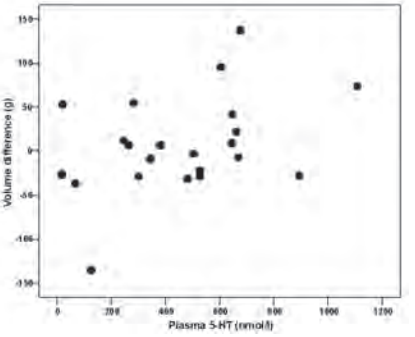

Figure 1E

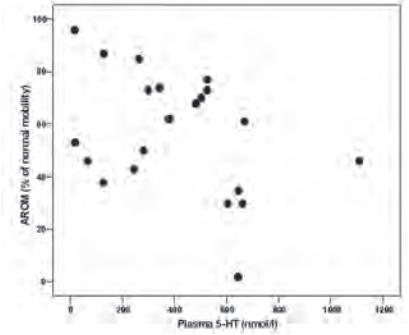

Figure 1F

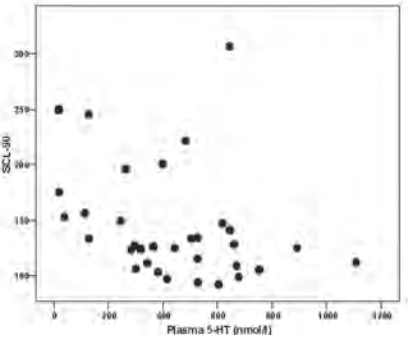

Figure 1. Distribution graphs of plasma 5-HT and VAS pain (A), plasma 5-HT and McGill pain questionnaire (B), plasma 5-HT and temperature difference (C), plasma 5-HT and volume difference (D), plasma 5-HT and AROM (E) and plasma 5-HT and SCL-90 questionnaire (F).

Discussion

Our results show that plasma 5-HT concentrations are an order of magnitude higher in CRPS1 patients than in an age-matched (not gender), healthy control group. However, we detected no significant correlation between 5-HT levels and pain, disease activity, and the results of a self-reported inventory.

The marked high 5-HT levels in platelet poor plasma (PPP) from CRPS1 patients are in the range normally found for platelet rich plasma (PRP). Platelets are considered to be the most important source of 5-HT in blood. So far no data have been presented regarding such high plasma 5-HT levels associated with a disease state. The simultaneously measured 5-HIAA plasma levels, which are in the normal range and even diminished in the CRPS1 patients, suggests a diminished breakdown of 5-HT in these patients.

The role of 5-HT in the course of CRPS1 remains unknown. Is 5-HT only one of a number of mediators formed in a cascade of processes, or should it be considered as one of a number of key mediators in the cascade of consecutive processes? In the search for other key mediators of the processes that follow the initial traumatic event, several bioactive substances have been suggested and investigated. Neuropeptides released after damage to nerve endings initiate the release of a cascade of vasoactive mediators [29]. In patients who have recently developed CRPS1, increased pro-inflammatory cytokines were found to be increased in skin blister fluid taken from the CRPS1 extremity in comparison with the contralateral extremity [30, 31]. After two years, skin blister fluid cytokine levels are still increased [32]; however, during the course of the disease a pronounced decline in cytokine levels is observed [33]. Other vasoactive mediators, such as nitric oxide [34] and presumably 5-HT, could play a role in the cell-cell interaction and activation of other mediators.

Based on the primary effects of 5-HT, in CRPS1 patients ischemia resulting in pain would be expected; furthermore, a diminished mobility, changing temperature and volume of the affected extremity could occur. Besides centrally affected pain perception, psychological dysfunction (depression) could have been developed. In patients with psychological dysfunction, mean plasma 5-HT levels in a range from 33 to 62 nmol/l have been reported; however, in some patients plasma 5-HT levels reached 200-250 nmol/l, similar to our observations [35]. In a recent study, plasma 5-HT levels of psychiatric patients and controls

were found to be 78 ± 58 nmol/l and 33 ± 38 nmol/l, respectively. Again, some individual patients showed levels of >150 nmol/l [36, 37].

From the pharmaceutical point of view, inhibition of 5-HT release from blood platelets and antagonizing the 5-HT2A receptor could counteract vasoactive effects associated with CRPS1 and promote recovery. This phenomenon has not been observed with therapies other than ketanserin. A beneficial effect of ketanserin on chronic peripheral burning pain has been suggested [12]. Furthermore, peripheral 5-HT2A receptor antagonism has been shown to attenuate primary thermal hyperalgesia and secondary mechanical allodynia following thermal injury [37]. However, this allodynia suppressing effect was attributed to the addition of L-carnitine, which affects ischemia and oxygen transport in the tissues, resulting in diminished pain intensity [38, 39]. In open studies, a number of CRPS1 patients have been treated with both ketanserin and L-carnitine [40]. That study suggests that pre-treatment with ketanserin is necessary to obtain the beneficial effects of L-carnitine. Alternatively, treating CRPS1 patients with NO donors could improve tissue blood distribution evoked by vasodilatation through increased cGMP levels [34]. Moreover, NO has shown to inhibit platelet 5-HT release [41]. Treatment with the antidepressant fluoxetine was associated with a significant decline in plasma 5-HT levels [42]. Until now, however, in CRPS1 patients only increased levels of catecholamines were associated with psychological dysfunction [43]. The role of 5-HT in psychological dysfunction [44] in CRPS1 patients still has to be confirmed.

In conclusion, the markedly elevated plasma 5-HT concentrations suggest a role for 5-HT in patients with CRPS1. However, because of the lack of correlations with distinct disease characteristics, 5-HT is probably one of a number of mediators in the cascade of consecutive processes that occur in the course of this painful and debilitating disease.

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References

1. Baron, R., J. Schattschneider, A. Binder, D. Siebrecht, and G. Wasner, Relation between sympathetic vasoconstrictor activity and pain and hyperalgesia in complex regional pain syndromes: a case-control study. Lancet, 2002. 359(9318): p. 1655-60.
2. Huygen, F.J., A.G. de Bruijn, J. Klein, and F.J. Zijlstra, Neuroimmune alterations in the complex regional pain syndrome. Eur J Pharmacol, 2001. 429(1-3): p. 101-13.
3. Raja, S.N. and T.S. Grabow, Complex regional pain syndrome I (reflex sympathetic dystrophy). Anesthesiology, 2002. 96(5): p. 1254-60.
4. Perez, R.S., W.W. Zuurmond, P.D. Bezemer, D.J. Kuik, A.C. van Loenen, J.J. de Lange, and A.J. Zuidhof, The treatment of complex regional pain syndrome type I with free radical scavengers: a randomized controlled study. Pain, 2003. 102(3): p. 297-307.
5. Coderre, T.J., D.N. Xanthos, L. Francis, and G.J. Bennett, Chronic post-ischemia pain (CPIP): a novel animal model of complex regional pain syndrome-type I (CRPS-I; reflex sympathetic dystrophy) produced by prolonged hindpaw ischemia and reperfusion in the rat. Pain, 2004. 112(1-2): p. 94-105.
6. Sasano, N., T. Tsuda, H. Sasano, S. Ito, K. Sobue, and H. Katsuya, A case of complex regional pain syndrome type II after transradial coronary intervention. J Anesth, 2004. 18(4): p. 310-2.
7. Silviu, B., W.J. Mark, I. Reuben, and P. Shvartzman, Complex Regional Pain Syndrome type I following radial artery cardiac catheterization. Int J Cardiol, 2005. 101(1): p. 167-8.
8. Houston, D.S. and P.M. Vanhoutte, Serotonin and the vascular system. Role in health and disease, and implications for therapy. Drugs, 1986. 31(2): p. 149-63.
9. Roberts, M.H., Involvement of serotonin in nociceptive pathways. Drug Des Deliv, 1989. 4(2): p. 77-83.
10. Bounameaux, H.M., H. Hellemans, and R. Verhaeghe, Ketanserin in chronic sympathetic dystrophy. An acute controlled trial. Clin Rheumatol, 1984. 3(4): p. 556-7.
11. Davies, J.A., T. Beswick, and G. Dickson, Ketanserin and guanethidine in the treatment of causalgia. Anesth Analg, 1987. 66(6): p. 575-6.
12. Hanna, M.H. and S.J. Peat, Ketanserin in reflex sympathetic dystrophy. A double-blind placebo controlled cross-over trial. Pain, 1989. 38(2): p. 145-50.
13. Q.I.H.i.t.N. (CBO), 2006. Evidence based guidelines (EBRO) complex regional pain syndrome type 1. ISBN-13: 978-90-8523-1240.
14. Reuben, S.S., Preventing the development of complex regional pain syndrome after surgery. Anesthesiology, 2004. 101(5): p. 1215-24.
15. Geertzen, J.H., A.T. de Bruijn-Kofman, H.P. de Bruijn, H.B. van de Wiel, and P.U. Dijkstra, Stressful life events and psychological dysfunction in Complex Regional Pain Syndrome type I. Clin J Pain, 1998. 14(2): p. 143-7.
16. Birkenhager, T.K., W.W. van den Broek, D. Fekkes, P.G. Mulder, P. Moleman, and J.A. Bruijn, Lithium addition in antidepressant-resistant depression: effects on platelet 5-HT, plasma 5-HT and plasma 5-HIAA concentration. Prog Neuropsychopharmacol Biol Psychiatry, 2007. 31(5): p. 1084-8.

17. Williams, J.L., F.N. Jacka, J.A. Pasco, S. Dodd, and M. Berk, Depression and Pain: an overview. Acta Neuropsychiatrica, 2006. 18: p. 79-87.
18. Bruehl, S., R.N. Harden, B.S. Galer, S. Saltz, M. Bertram, M. Backonja, R. Gayles, N. Rudin, M.K. Bhugra, and M. Stanton-Hicks, External validation of IASP diagnostic criteria for Complex Regional Pain Syndrome and proposed research diagnostic criteria. International Association for the Study of Pain. Pain, 1999. 81(1-2): p. 147-54.
19. Bax, W.A., G.J. Renzenbrink, E.A. van der Linden, F.J. Zijlstra, D. van Heuven-Nolsen, D. Fekkes, E. Bos, and P.R. Saxena, Low-dose aspirin inhibits platelet-induced contraction of the human isolated coronary artery. A role for additional 5-hydroxytryptamine receptor antagonism against coronary vasospasm? Circulation, 1994. 89(2): p. 623-9.
20. Carlsson, A.M., Assessment of chronic pain. I. Aspects of the reliability and validity of the visual analogue scale. Pain, 1983. 16(1): p. 87-101.
21. Merskey, H. and N. Bogduk, Classification of chronic pain: descriptions of chronic pain syndromes and definitions of pain terms. 1994, IASP Press: Seattle, WA. p. 40-43.
22. Lowe, N.K., S.N. Walker, and R.C. MacCallum, Confirming the theoretical structure of the McGill Pain Questionnaire in acute clinical pain. Pain, 1991. 46(1): p. 53-60.
23. Hershler, C., T.A. Conine, A. Nunn, and M. Hannay, Assessment of an infra-red non-contact sensor for routine skin temperature monitoring: a preliminary study. J Med Eng Technol, 1992. 16(3): p. 117-22.
24. Fereidoni, M., A. Ahmadiani, S. Semnanian, and M. Javan, An accurate and simple method for measurement of paw edema. J Pharmacol Toxicol Methods, 2000. 43(1): p. 11-4.
25. Oerlemans, H.M., R.A. Oostendorp, T. de Boo, and R.J. Goris, Evaluation of three methods to rate impairment in patients with complex regional pain syndrome I of one upper extremity. Clin Rehabil, 2000. 14(3): p. 331-9.
26. Perez, R.S., H.M. Oerlemans, W.W. Zuurmond, and J.J. De Lange, Impairment level SumScore for lower extremity Complex Regional Pain Syndrome type I. Disabil Rehabil, 2003. 25(17): p. 984-91.
27. Kemler, M.A., C.P. Rijks, and H.C. de Vet, Which patients with chronic reflex sympathetic dystrophy are most likely to benefit from physical therapy? J Manipulative Physiol Ther, 2001. 24(4): p. 272-8.
28. Arrindell, W.A. and H. Ettema, The Dutch version of the Symptom Checklist (SCL-90). Ned Tijdsch Psych (Dutch J Psychol), 1981. 36: p. 77-108.
29. Birklein, F., M. Schmelz, S. Schifter, and M. Weber, The important role of neuropeptides in complex regional pain syndrome. Neurology, 2001. 57(12): p. 2179-84.
30. Matsumura, H., Y. Jimbo, and K. Watanabe, Haemodynamic changes in early phase reflex sympathetic dystrophy. Scand J Plast Reconstr Surg Hand Surg, 1996. 30(2): p. 133-8.
31. Heijmans-Antonissen, C., F. Wesseldijk, R.J. Munnikes, F.J. Huygen, P. van der Meijden, W.C. Hop, H. Hooijkaas, and F.J. Zijlstra, Multiplex bead array assay for detection of 25 soluble cytokines in blister fluid of patients with complex regional pain syndrome type 1. Mediators Inflamm, 2006(1): p. 28398.
32. Munnikes, R.J., C. Muis, M. Boersma, C. Heijmans-Antonissen, F.J. Zijlstra, and F.J. Huygen,

Intermediate stage complex regional pain syndrome type 1 is unrelated to proinflammatory cytokines. Mediators Inflamm, 2005(6): p. 366-72.

33. Wesseldijk, F., F.J. Huygen, C. Heijmans-Antonissen, S.P. Niehof, and F.J. Zijlstra, Tumor necrosis factor-alpha and interleukin-6 are not correlated with the characteristics of Complex Regional Pain Syndrome type 1 in 66 patients. Eur J Pain, 2008. 12(6): p. 716-21.
34. Groeneweg, J.G., F.J. Huygen, C. Heijmans-Antonissen, S. Niehof, and F.J. Zijlstra, Increased endothelin-1 and diminished nitric oxide levels in blister fluids of patients with intermediate cold type complex regional pain syndrome type 1. BMC Musculoskelet Disord, 2006. 7: p. 91.
35. Spreux-Varoquaux, O., J. Gailledreau, B. Vanier, D. Bothua, J. Plas, J.F. Chevalier, C. Advenier, M. Pays, and S. Brion, Initial increase of plasma serotonin: a biological predictor for the antidepressant response to clomipramine? Biol Psychiatry, 1996. 40(6): p. 465-73.
36. Tyano, S., G. Zalsman, H. Ofek, I. Blum, A. Apter, L. Wolovik, L. Sher, E. Sommerfeld, D. Harell, and A. Weizman, Plasma serotonin levels and suicidal behavior in adolescents. Eur Neuropsychopharmacol, 2006. 16(1): p. 49-57.
37. Sasaki, M., H. Obata, K. Kawahara, S. Saito, and F. Goto, Peripheral 5-HT2A receptor antagonism attenuates primary thermal hyperalgesia and secondary mechanical allodynia after thermal injury in rats. Pain, 2006. 122(1-2): p. 130-6.
38. Lopaschuk, G., Regulation of carbohydrate metabolism in ischemia and reperfusion. Am Heart J, 2000. 139(2 Pt 3): p. S115-9.
39. Hiatt, W.R., J.G. Regensteiner, M.A. Creager, A.T. Hirsch, J.P. Cooke, J.W. Olin, G.N. Gorbunov, J. Isner, Y.V. Lukjanov, M.S. Tsitsiashvili, T.F. Zabelskaya, and A. Amato, Propionyl-L-carnitine improves exercise performance and functional status in patients with claudication. Am J Med, 2001. 110(8): p. 616-22.
40. Moesker, A., Complex regional pain syndrome, formerly called reflex sympathetic dystrophy, treatment with ketanserin and carnitene. 2000, Thesis, Erasmus MC.
41. Juhasz, G., T. Zsombok, E.A. Modos, S. Olajos, B. Jakab, J. Nemeth, J. Szolcsanyi, J. Vitrai, and G. Bagdy, NO-induced migraine attack: strong increase in plasma calcitonin gene-related peptide (CGRP) concentration and negative correlation with platelet serotonin release. Pain, 2003. 106(3): p. 461-70.
42. Alvarez, J.C., N. Gluck, A. Fallet, A. Gregoire, J.F. Chevalier, C. Advenier, and O. Spreux-Varoquaux, Plasma serotonin level after 1 day of fluoxetine treatment: a biological predictor for antidepressant response? Psychopharmacology (Berl), 1999. 143(1): p. 97-101.
43. Harden, R.N., N.J. Rudin, S. Bruehl, W. Kee, D.K. Parikh, J. Kooch, T. Duc, and R.H. Gracely, Increased systemic catecholamines in complex regional pain syndrome and relationship to psychological factors: a pilot study. Anesth Analg, 2004. 99(5): p. 1478-85; table of contents.
44. Fekkes, D., L. Timmerman, and L. Pepplinkhuizen, Effects of clomipramine on plasma amino acids and serotonergic parameters in panic disorder and depression. Eur Neuropsychopharmacol, 1997. 7(3): p. 235-9.

Chapter 6

Multiplex-25 Bead Array Assay for detection of soluble cytokines in blister fluid and plasma of patients with CRPS type 1

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Abstract
Inflammatory processes are known to be involved at least in the early phase of complex regional pain syndrome type 1 (CRPS1). Blister fluid obtained from the involved extremities displayed increased amounts of pro-inflammatory cytokines IL-6 and TNFα compared with the non-involved extremities. The aim was to investigate the involvement of mediators by measurement of several other cytokines using new detection techniques that enable multiple cytokine measurement in small samples. The use of a multiplex-25 bead array cytokine assay and LuminexTM technology enabled simultaneous measurement of representative 1) pro-inflammatory cytokines such as GM-CSF, IL-1β, IL-1RA, IL-6, IL-8 and TNF-α; 2) Th1/Th2 distinguishing cytokines IFN-γ, IL-2, IL-2R, IL-4, IL-5 and IL-10; 3) non-specific acting cytokines IFN-α, IL-7, IL-12p40/p70, IL-13, IL-15 and IL-17; and 4) chemokines Eotaxin, IP-10, MCP-1, MIP-1α, MIP-1β, MIG and RANTES. Although minimal detection levels are significantly higher in the bead array system than those in common ELISA assays, in blister fluid IL-1RA, IL-6, IL-8, TNF-α, IL-12p40/p70, MCP-1 and MIP-1β were detectable and increased in CRPS1 affected extremities. Levels of IL-6 and TNF-α simultaneously measured by ELISA (Sanquin Compact kit) and by multiplex-25 bead array assay (Biosource) were highly correlated ($r = 0.85$, $P < 0.001$ for IL-6 and $r = 0.88$, $P < 0.001$ for TNF-α). Furthermore, IP-10 and eotaxin were detectable but diminished in CRPS1, whereas detectable amounts of IL-10 were similar in involved and non-involved extremities. In conclusion, multiplex bead array assays are useful systems to establish the involvement of cytokines in inflammatory processes by measurements in blister fluids of CRPS1. Ten representative cytokines were detectable. However, detection levels and amounts measured are at least 3 times higher in the multiplex-25 array assay than in the ELISA assays used simultaneously for the measurement of cytokines.

Introduction
Complex regional pain syndrome type 1 (CRPS1), also known as reflex sympathetic dystrophy (RSD), is a debilitating painful disease in an extremity that is characterised by signs of allodynia and hyperalgesia, as well as vasomotor, sudomotor and motor trophic signs and symptoms. In general the disease persists in one extremity [1, 2]. The diagnosis of CRPS1 is mainly based

on clinical observation [3, 4], for which international research criteria have been determined [5]. Although some patients develop CRPS1 after an inciting event (trauma or surgery in the hand, foot or knee), the origin of this invalidating disease remains unknown. Subgroups of CRPS1 patients are described in whom either vasomotor signs, neuropathic pain or all signs of inflammation are prominent factors [6]. Studies on the underlying mechanisms of this disease have ranged from the effects of physiotherapy to pharmaceutical intervention and from biological active mediators to genetic mapping. During the initial stage of the disease most symptoms, such as oedema, redness, loss of function and temperature changes [7], suggest a local inflammatory process [8]. Therefore we subsequently investigated the involvement of inflammatory mediators during the initial stage of this disease and showed that the cytokines interleukin-6 (IL-6) and tumour necrosis factor α (TNF-α) were significantly increased in the affected hand or foot [9], which was confirmed by other markers of inflammation [10]. Most treatments of CRPS1 are not evidence based. The patient-dependent choice of either physical therapy, pharmaceutical intervention or unconventional alternative medicine is still a matter of debate [8, 11]. Targeted treatment with anti-TNF (Infliximab) seems, however, to be successful in patients with confirmed signs of inflammation [12].

In all our recent studies, skin blister fluids showed elevated amounts of IL-6 and TNF-α as a measure of local inflammation intensity. Due to the limited amount of fluid, however, in the same sample we were only able to measure 2 or 3 different mediators separately. Therefore the present study aimed to confirm the involvement of inflammatory processes underlying CRPS1 by measuring a large variety of cytokines simultaneously in the same small blister fluid sample.

Until now commercially available ELISA kits are used to measure levels of cytokines in biological samples. Most of these kits require a two-fold diluted sample volume of 100 µl. Therefore, to examine a number of different classes of cytokines, volumes of more than a few hundred µl should be available, otherwise dilutions need to be made. However, this process of dilution could result in values that are below the detectable standard. The simultaneous measurement of a number of cytokines in a single sample using a new developed microbead-based flow cytometry system (LuminexTM), enables to detect of cytokines in small volume

samples of human biological material [13].
Successful measurement of six Th1/Th2 cell distinguishing cytokines (interferon-γ (IFN-γ), TNF-α, IL-2, IL-4, IL-5 and IL-10) have been reported in a single sample of human tears obtained from allergic patients [14, 15], and in plasma from children with neonatal sepsis; in these newborn infants, in the same samples the contribution of inflammatory cytokines (IL-1β, IL-6, IL-8, IL-10, IL-12 and TNF-α) was also evaluated [16]. This so-called inflammation panel was also used for the simultaneous measurement of cytokines in tracheal aspirates after mechanical ventilation [17].
Here we report on the simultaneous detection of 25 cytokines in blister fluids obtained from both the involved and the non-involved (contra lateral) extremities of CRPS1 patients. This is the first study in which such a large number of inflammatory cytokines, Th1/Th2 distinguishing cytokines and chemokines have been investigated in human skin blister fluids.

Methods and materials

Patients and blister fluids

For this study 22 patients (4 male, 18 female; mean age 52 ± 8.2 (SD) years) were selected, with a mean duration of the disease of 2.75 ± 1.25 (SD) years, all being in the intermediate phase. During CRPS1 we in general distinguish four different disease phases. Stage I is defined as the warm or hypertrophic phase, stage II is defined as the intermediate phase, stage III is defined as the cold or early chronic phase, and at last in stage IV the definite chronic phase corresponds to atrophic signs, dystonie, and per definition stabilization of the disease or, in rare instances, to healing [8-12, 18].
All 22 patients were characterized using the impairment sum score (ISS, according to Oerlemans et al [7].). At the time of the study this was 38 ± 16.6 (SD) on a scale of 0-100, indicating a medium disease activity. This score was calculated based on differences in skin surface temperature, volume of oedema, quantity of pain (Visual Analogue Scale), intensity of pain (McGill pain questionnaire) and motor function (as Active Range of Motion).
Blisters were induced by means of a suction method [9, 10]. A 3-hole (5 mm diameter per hole) skin suction chamber was positioned on the skin of the upper extremity, on the dorsal side

of the involved hand and the flexor side of the non-involved forearm.
A vacuum of 300 mm Hg negative pressure was applied with an Atmoforte 350A aspirator pump (ATMOS Medizintechnik, Lenzkirch, Germany), which was reduced after 15 minutes to 250 mm Hg and again, 15 minutes later, reduced to 200 mm Hg. This negative pressure was maintained until blisters containing sufficient fluid had been developed, but not longer than 2.5 hours. The contents of the blisters were punctured and pooled from each side into a 1.5 ml Eppendorff conical polypropylene tube and centrifuged for 5 minutes at 1600 xg. The mean recovery of supernatants from control blisters was 173 ± 21 (± SEM) µl fluid, and 168 ± 17 µl blister fluid from the CRPS1 side. All samples were stored in 1 ml conical polypropylene tubes at –80 °C until analysis [9].
In these blister fluid samples IL-6 and TNF-α were analyzed separately by ELISA and a set of 25 cytokines were analyzed simultaneously using the LuminexTM system and the Multiplex-25 array assay from Biosource.

Enzyme-Linked Immunosorbent Assays
Blister samples were diluted 4-fold in appropriate calibrator diluent assay buffer for the direct measurement of cytokines. Cytokine assays were performed following the manufacturers protocol (Pelikine™ human ELISA compact kits for IL-6 (M1906) and TNF-α (M1920), Sanquin, Amsterdam, The Netherlands). The standard curve ranges and mean calculated zero signal plus 3 SD for IL-6 were 0-450 pg/ml and 0.2 pg/ml, respectively; and for TNF-α 0-1000 pg/ml and 1 pg/ml, respectively. The requested solutions were provided with the ELISA compact kits and additional toolkits (Pelikine-ToolTM set (M1980), Sanquin, Amsterdam, The Netherlands).
In brief, the ELISA procedure (performed at room temperature) was as follows: The wells of a 96-wells plate were pre-coated overnight with 100 µl of coating antibody, diluted 1:100 with coating buffer (0.1 M carbonate/bicarbonate). Thereafter the wells were washed 5 times with 400 µl of phosphate buffered saline (PBS) containing 0.005% TWEEN and then blocked with 200 µl of blocking buffer (1:20 diluted in PBS) for 1 hour on a shaker. After washing the plate five times with washing buffer, 100 µl of unknown blister fluid samples (diluted 1:4 in assay dilution buffer) or standards were pipetted into the wells. The plate was incubated

for 1 hour on a shaker. After washing the plate five times with washing buffer, 100 µl of biotinylated antibody (diluted 1:100 in assay dilution buffer) was pipetted into the wells and incubated 1 hour on the shaker. After washing the plate, the wells were incubated 30 minutes on a shaker with 100 µl of streptavidin-HRP conjugate (diluted 1:10,000 in assay dilution buffer). Thereafter the plate was washed for the last time with washing buffer and incubated with 100 µl of tetramethylbenzidine substrate solution. The reaction was stopped after 30 minutes with 100 µl of stop solution (1.8 M2SO4). The absorbance per well was measured at 450 nm with a Medgenix ELISA reader. Sample concentrations were calculated using the appropriate standard calibration lines and the Softmax® software of the reader.

Multiplex–25 bead array assay

The human Cytokine multiplex-25 bead array assay kit for LuminexTM was purchased from Biosource (Nivelles, Belgium). This kit comprises all components necessary for the whole assay procedure, to be fulfilled within approximately 5 hours hands-on time.

The following cytokines could be measured:

- Inflammatory panel: GM-CSF (granulocyte, macrophage–colony stimulating factor), IL-1β, IL-1RA (interleukin-1 Receptor Antagonist), IL-6, IL-8, TNF-α;
- Th1/Th2 panel: IFN-γ, IL-2, IL-2R, IL-4, IL-5, IL-10;
- Cytokine II panel: IFN-α, IL-7, IL-12p40/p70, IL-13, IL-15, IL-17;
- Chemokine panel: Eotaxin, IP-10 (interferon-γ inducing protein, 10 kDa), MCP-1 (monocyte chemotactic protein), MIP-1α (macrophage inflammatory protein) , MIP-1β, MIG (monokine induced by γ-interferon), RANTES (Regulated upon Activation Normal T cell Expressed and Secreted).

Standard curves for each cytokine (in duplicate) were generated by using the reference cytokine concentrations supplied in this kit. Blister samples were diluted 4-fold in appropriate assay diluent. The assay was performed in a 96-well filter plate, using all the assay components provided in the kit. All incubation steps were performed at room temperature and in the dark to protect the beads from light.

In brief, the following procedure was performed: Firstly, the filter plate was pre-wetted with 200 µl of working washing solution

and then this solution was aspirated from the wells using a vacuum manifold. The beads (25 µl) were pipetted into each well and thereafter the filter plate wells were washed two times with washing buffer using the vacuum manifold. Incubation buffer (50 µl) and 1:4 diluted blister fluid samples or standards (50 µl) were pipetted into the wells and incubated for 2 hours with the beads. Thereafter the wells were washed using the vacuum manifold and detector antibody conjugated to biotin (diluted 1:10 with biotin diluent) was added. After incubation for 1 hour, beads were washed again followed by an incubation of 30 minutes with streptavidin conjugated to the fluorescent protein, R-Phycoerythrin (streptavidin-RPE, diluted 1:10). After washing to remove the unbound streptavidin-RPE, the beads (minimum of 50 beads per cytokine) were analysed in the LuminexTM 100 instrument (Applied Cytometry Systems, Dinnington, UK), which monitored the spectral properties of the beads while simultaneously measuring the amount of fluorescence associated with R-phycoerythrin. Raw data (mean fluorescence intensity, MFI) were analyzed using StarStationTM software (Applied Cytometric Systems, Dinnington, UK).

Statistical Analysis

All cytokines showed a skewed distribution. Comparison of paired samples (CRPS1 versus non-involved extremity) was performed with the paired t-test after logarithmic transformation of the data obtained from measurements in blister fluids. In case of values below the detection limit, the outcome was set at the detection limit and the paired sample t-test with adjustment for these left-censored values, was performed using STATA software (CNREG procedure). The same method was used to assess the assumed linear relation shown in Figure 1. Correlation coefficients were determined by the Spearman's test for untransformed data.

Results

To our knowledge we are the only clinical investigators reporting on cytokine levels in blister fluids obtained from CRPS1 patients [9, 10, 19]. Therefore we searched for data obtained from artificial blisters in immunological skin diseases in order to compare detection ranges (Table 1).

Table 1: Literature overview of cytokine levels in blister fluid measured by ELISA

Disease	Refs		IL-1β (pg/ml)	IL-6 (pg/ml)	IL-8 (pg/ml)	TNF-α (pg/ml)	IL-4 (pg/ml)	IL-10 (pg/ml)
Complex regional pain syndrome	9,10	involved	≤ 2	54	-	31	-	-
		non-involved	≤ 2	6	-	8	-	-
Psoriasis	33,34	involved	122	1683	-	145	-	-
		non-involved	≤ 3	121	-	9	-	-
Psoriasis	35	involved	-	870	-	195	-	-
		non-involved	-	423	-	84	-	-
Epidermal necrolysis	36	involved	73 m	66	10	-	-	33
		non-involved	2 m	ND	ND	-	-	ND
Buallous pemphigus	21,37	involved	-	245 m	-	-	9	54
		non-involved	-	16 m	-	-	≤ 4	≤ 5
Bullous pemphigus	38	involved	-	-	-	-	-	73
		non-involved	-	-	-	-	-	ND
Pemphigus vulgaris	39	involved	-	-	-	-	-	186 m
		non-involved	-	-	-	-	-	ND

Data are medians or otherwise means (indicated by m)
ND: not determined

ELISA

Both IL-6 and TNF-α were measured in blister fluid samples of 22 CRPS1 patients, obtained from both the involved and the non-involved extremity. Standards were measured in duplicate for 8 data points including a zero standard. Standard curves were plotted through four parameter logistic curve fitting. R-squared values were 0.999 and 1.00, respectively. Calculated levels are presented in Table 2a. Because cytokines displayed a not normally distributed set of data, the median and the ranges are presented. Interleukin-6 and TNF-α were significantly increased at the CRPS1 side (paired sample test).

Table 2a: Cytokine levels in blister fluids from 22 patients with complex regional pain syndrome measured by ELISA

ELISA	lowest detectable level (pg/ml)	levels in blister fluid median (range) in pg/ml non-involved	CRPS1	P-value
		Inflammatory panel		
IL-6	0.2	2.7 (≤ 0.8–191)	38 (≤ 0.8–346)	0.002
TNF-α	0.5	10.3 (2.1-315)	48 (2.8-381)	0.006

Multiplex

Twenty-five cytokines were measured in the same blister fluid samples of the 22 CRPS1 patients as indicated in the previous section 'ELISA'. Cytokine-specific single beads (25 different bead populations) were identified through sequential gating on Doublet Discriminator signal and intrinsic bead dye (red vs. infra-red) excluding bead aggregates and debris. The amount of cytokine was measured as mean fluorescence intensity (MFI) of the streptavidin-RPE signal on the outside of the beads from a minimum of 50 beads per cytokine. Standards were measured in duplicate for 9 data points including a zero standard.

Standard curves were plotted through four or five parameter logistic curve fitting. All R-squared values were between 0.99 and 1.00, except for IL-7 (0.968).

In blister fluid from the 'inflammatory panel' IL-1RA, IL-6, IL-8 and TNF-α were detectable, from the 'cytokine II panel' IL-12p40 was detectable and from the 'chemokine panel' MCP-1 and MIP-1β were detectable and all were increased in CRPS1 affected extremities.

Table 2b: Cytokine levels in blister fluids from 22 patients with complex regional pain syndrome measured by multiplex-25 bead array assay

25-plex	lowest detectable level (pg/ml)	levels in blister fluid median (range) in pg/ml non-involved	CRPS1	P-value
		Inflammatory panel		
GM-CSF	11	all ≤ 44	all ≤ 44	nt
IL-1β	12	all ≤ 48	all ≤ 48	nt
IL-1RA	50	35940 (12665-67549)	48894 (23393-90714)	< 0.001
IL-6	2	≤ 8 (≤ 8–100)	100 (≤ 8–2055)	0.001
IL-8	7	≤ 28 (≤ 28-301)	46 (≤ 28-519)	0.006
TNF-α	9	≤ 36 (≤ 36-829)	195 (≤ 36-1923)	0.013
		Th1/Th2 panel		
IFN-γ	3	all ≤ 12	all ≤ 12	nt
IL-2	4	all < 16	all ≤ 16	nt
IL-2R	30	all ≤ 120	all ≤ 120	nt
IL-4	2	all ≤ 8	all ≤ 8	nt
IL-5	2	all ≤ 8	all ≤ 8	nt
IL-10	4	20 (≤ 16-51)	21 (≤ 16-50)	0.336
		Cytokine II panel		
IFN-α	10	all ≤ 40	all ≤ 40	nt
IL-7	28	all ≤ 112	all ≤ 112	nt
IL-12p40	4	325 (192-540)	386 (256-542)	0.007
IL-13	3	all ≤ 12	all ≤ 12	nt
IL-15	6	all ≤ 24	all ≤ 24	nt
IL-17	6	all ≤ 24	all ≤ 24	nt
		Chemokine panel		
Eotaxin	3	29 (15-54)	24 (≤ 12-55)	0.009
IP-10	3	48 (24-185)	37 (≤ 12-137)	0.025
MCP-1	3	297 (126-1570)	579 (188-4415)	0.002
MIP-1α	10	all ≤ 40	all ≤ 40	nt
MIP-1β	10	199 (116-450)	290 (135-557)	0.001
MIG	12	all ≤ 48	all ≤ 48	nt
RANTES	10	all ≤ 40	all ≤ 40	nt

Blister fluids were diluted 4-fold in matrix buffer.
Lowest detectable level: lowest detectable standard which significantly differs from zero standard (experimentally determined).
P-values: nt: not tested, because all measured outcomes were below detection level.

Furthermore, from the 'chemokine panel' IP-10 and Eotaxin were detectable and diminished in CRPS1, whereas from the'Th1/Th2 panel' detectable amounts of IL-10 were similar in both extremities (Table 2b).

Statistical considerations

An analysis by using non-parametric statistics (Wilcoxon's signed rank test), with outcomes set at the lower limit of detections in case of values below this limit, resulted in similar P-values for all parameters. We did not adjust for multiple comparison tests because our study had an exploratory character.

Comparison of the two methods

Levels of IL-6 and TNF-α measured by ELISA and by the multiplex-25 bead array assay were highly correlated ($r = 0.85$, $P < 0.001$ for IL-6, Figure 1a, and $r = 0.88$, $P < 0.001$ for TNF-α, Figure 1b).

In the multiplex-25 bead array assay for IL-6 17 of 44 samples were not detectable (≤ 8 pg/ml), whereas for TNF-α 20 of 44 samples were not detectable (≤ 36 pg/ml). In the IL-6 ELISA only one sample was below the detection level.

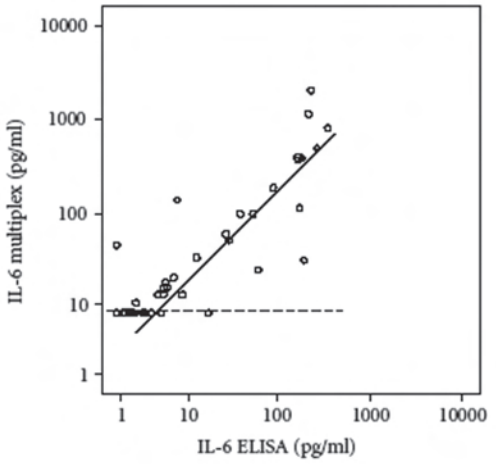
Correlations of multiplex-25 measured cytokines

The cytokines IL-6, IL-8, IL10, IL-12, TNF-α, MIP-1β and MCP-1 were significantly correlated with each other (Table 3), whereas IL-12p40 was only (highly) correlated with IL-1RA (Figure 2).

Table 3: Nonparametric correlations of cytokines in blister fluid from CRPS1 hand

	IL-8	IL-10	IL-12	TNF-α	MIP-1β	MCP-1
IL-1RA			0.94a			
IL-6	0.85a	0.56c	0.51d	0.78a	0.72a	0.88a
IL-8		0.63b	0.44e	0.93a	0.76a	0.88a
IL-10				0.56c	0.62b	0.59b
IL-12				0.48e	0.50d	0.47e
TNF-α					0.67b	0.81a
MIP-1						0.68e

P-values: a < 0.001, b < 0.005, c < 0.01, d < 0.02, e < 0.05



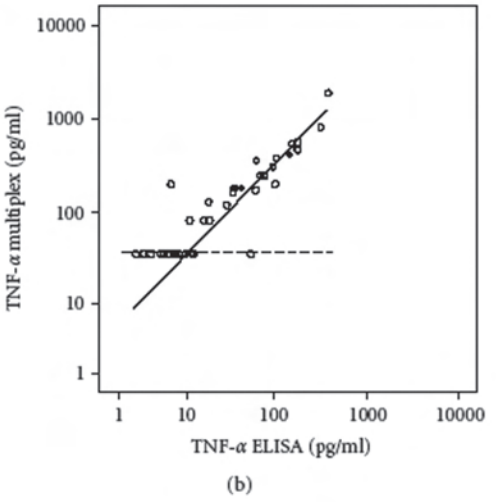


Figure 1: Regression curves of 44 samples of blister fluid obtained from 22 CRPS1 patients, both from the involved and the non-involved extremity. Values calculated in pg/ml were plotted on logarithmic scales. Regression lines were calculated taking into account the left-censored values due to detection limits as described in the statistical methods. Dotted lines indicate detection levels of the multiplex-25 cytokine assay. 1a: Regression curve of IL-6 data from the multiplex-25 cytokine assay (Biosource) and the ELISA kit (Sanquin); ($r = 0.85$, $P < 0.001$). 1b: Regression curve of TNF-α data from the multiplex-25 cytokine assay (Biosource) and the ELISA kit (Sanquin); ($r = 0.88$, $P < 0.001$).

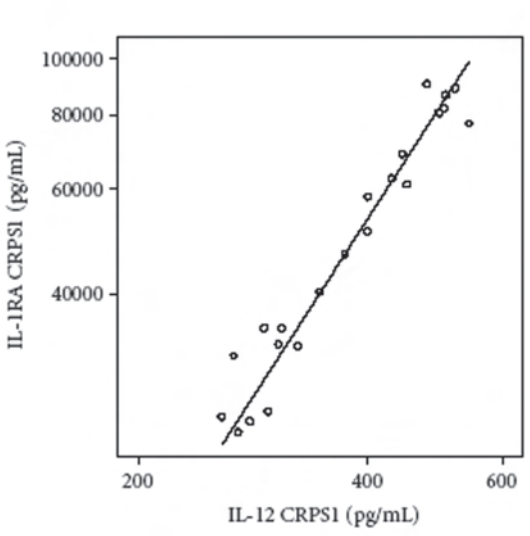


Figure 2: Example of a regression curve between concentrations of IL-1RA and IL-12 in 22 blister fluid samples taken from the CRPS1 extremity (correlation coefficient 0.97, $P < 0.001$), measured by the multiplex-25 bead array assay. Values are calculated in pg/ml and plotted on a logarithmic scale.

Discussion

This study investigated the involvement of inflammatory mediators in CRPS1 represented by a large variety of cytokines simultaneously measured in one small blister fluid sample. Use of the new multiplex-25 bead array assay allowed to determine 10 cytokines in blister fluid samples, considerably above the relatively high detection levels of these integrated cytokine assays.

Inflammatory panel

In our earlier observations of mediators in blister fluid of CRPS1 patients (reflecting inflammation at the affected extremity), we decided to measure both IL-6 and TNF-α as representative markers of inflammation [9, 10, 19]. At that time the amounts of cytokines we found were within the ranges reported for other (mainly dermatological) diseases (Table 1). The present results obtained after simultaneous measurement of cytokines by the multiplex-25 array assay were (although 2-3 times higher) in the same range. In those 44 blister fluid samples high correlations were found between data obtained from ELISA measurements

and the multiplex-25 bead array assays (Figs. 1a and 1b).
In addition to these findings, significant amounts of other (pro-) inflammatory cytokines were detectable in blister fluid by the multiplex-25 bead array assay, namely IL-8 and IL-12p40/p70. Furthermore, relatively high amounts of IL-1RA were found. Amounts of IL-1RA were comparable to those found by Blaha et al [20].
Using the multiplex-25 bead array assay, GM-CSF was not detectable. This was, however, also the case in blister fluid of patients with bullous pemphigus (BP), a chronic auto-immune blistering disease, in which GM-CSF was not detectable (< 5 pg/ml) by ELISA [21].

Th1/Th2 panel
In the present study we concluded that the cytokines IFN-γ, IL-2, IL-2R, IL-4, IL-5 and IL-10 normally involved in the Th1/Th2 pathways were negligible, because all calculated data were around or beneath the detection limits of the multiplex-25 bead array assay.
Detectable amounts of IL-4 and IL-10 (Table 1) and of soluble IL-2R have been measured in the blister fluid of patients with toxic epidermal necrolysis, a disease in which the early participation of activated CD8+ T lymphocytes play an important role [22].

Chemokines
Eotaxin and IL-5 are representative chemotactic cytokines to study the activation of skin-homed eosinophils, which in general represent allergic reactions [23]. In pemphigoid gestationis, a rare autoimmune bullous disease of late pregnancy, both markers are significantly increased in blister fluid [24]. In another study, elevated levels of both eotaxin and IL-5 in blister fluid of BP were found, suggesting tissue eosinophilia [25].
In the present study IL-5 was not detectable, although low detection ranges were achieved. On the contrary, eotaxin was detectable at levels > 12 pg/ml blister fluid, but was surprisingly decreased in CRPS1 blister fluid. Therefore, we concluded that allergic reactions do not play an important role in CRPS1.
In our study, both MCP-1 and MIP-1β were present in blister fluid in significant amounts and were increased in CRPS1 blisters in comparison with non-involved blister samples, suggesting an

ongoing involvement of activated monocytes and macrophages. In blisters generated in skin of chronic ambulatory peritoneal dialysis patients, however, MCP-1 concentrations in this interstitial fluid were not related to the intensity of the inflammation [26, 27].

Source of cytokines

The involvement, cellular sources and most prominent effects of cytokines in BP, partly detected in blister fluids, have been reviewed extensively [28, 29]. In our observations, a number of detectable mediators measured at the CRPS1 side were correlated individually, except for IL-1RA, eotaxin and IP-10 (Table 4). Our data suggest that detectable mediators have been generated by a homogenous cell population. Because T-cells apparently are not involved, the most likely candidates are monocytes, macrophages and possibly fibroblasts. The main products generated by these cells are IL-6, IL-8, IL-10, IL-12 and TNF-α. Apparently, skin mast cells are also involved, as reflected by increased amounts of tryptase in CRPS1 blister fluid [10]. The main cytokine produced by mast cells is TNF-α.

The amount of cytokines IL-8, IL-6, MCP-1, GM-CSF, TNF-α and MIP-1β, secreted by human epithelial cells from the female reproductive tract was recently assessed by Luminex bead array analysis [30]. The main products found were IL-8 and IL-6, but these were 100-fold higher than those of GM-CSF, TNF-α and MIP-1β. Therefore, regarding the distribution of our data, it is unlikely that epithelial cells contributed to the levels of cytokines found in blister fluid of CRPS1 patients.

Sensitivity of multiplex-25 bead array assay

The sensitivity of multiplex bead array assays for the detection of soluble cytokines and the quantitative values from several manufacturers have been compared for serum samples [31]. Bead array and ELISA values appeared to be comparable between the manufacturers. The minimal detection range for the BioSource kit was comparable with the R&D Systems assay kit, but about 2-fold and 5-fold higher than kits from Bio-Rad and LINCO Research, respectively. The simultaneous measurement of 15 human cytokines (Bio-Plex system from Bio-Rad) in a single sample of cultured peripheral blood mononuclear cells compared with regular ELISA kits (purchased from a number of manufac-

turers), resulted in high correlation coefficients ranging from 0.75 to 0.99 [32]. Our comparison between the multiplex-25 bead array assay (Biosource) and ELISAs (Sanquin) for IL-6 and TNF-α also revealed high correlation coefficients, ranging from 0.85 to 0.88 (Figure 1).

The high detection levels in the present study were partly caused by the 4-fold dilutions needed to enable separate determinations by ELISA techniques. Generally, at least 50 µl will be recovered per blister evoked by suction. After a 2-fold dilution in assay matrix buffer a duplicate measurement by the multiplex bead array assay could be performed. Then detection levels will be more acceptable; however, they will still be 3-fold (or even more) increased compared with the commonly used ELISAs. In case of paired sample measurements (involved vs. non-involved) or treatment-affected paired sample measurements, these shortcomings are acceptable for the selected cytokine panels as demonstrated by the results of this study.

We failed to detect substantial amounts of protein in blister fluid of at least 15 selected cytokines assayed in this multiplex-25 array system, although the detection of some of these cytokines (such as IL-1β, IL-2, IL-5, IL-7, IL-15, IFN-γ and RANTES) has been realised in blister fluid using commonly available ELISA kits which are more sensitive [28]. Based on these ELISA derived data, the levels of cytokines detectable in blister fluids taken from a variety of diseases, generally were above our detection limits. Therefore, we concluded that these cytokines are not prominent mediators involved in CRPS1.

Conclusion

Based on our findings, routine application of a multiplex-25 bead array assay to detect representative cytokines in blister fluids would not be advisable. The use of this system is advisable for investigational purposes or for diagnosis based on selected cytokines from relevant literature. We therefore propose that a selection of two or three representatives from each panel (the inflammatory cytokines panel, the Th1/Th2 cytokines panel and the chemokines panel) would be sufficient to indicate the actvity of the CRPS1 disease. During the course of the disease this selected panel could also be used to indicate the effectiveness of therapeutic intervention. Based on our data and the selection made by Fahey et al [30], we suggest to include at least IL-6, IL-8, TNF-α, MCP-1, MIP-1β,

IL-10 and IL-12 in that investigation panel.
Future research using blister fluid should also focus on standardisation of the blister techniques and the warranted inclusion of control samples, either from non-involved tissue or from healthy volunteers.

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References

1. Baron, R., J. Schattschneider, A. Binder, D. Siebrecht, and G. Wasner, Relation between sympathetic vasoconstrictor activity and pain and hyperalgesia in complex regional pain syndromes: a case-control study. Lancet, 2002. 359(9318): p. 1655-60.
2. Raja, S.N. and T.S. Grabow, Complex regional pain syndrome I (reflex sympathetic dystrophy). Anesthesiology, 2002. 96(5): p. 1254-60.
3. Veldman, P.H., H.M. Reynen, I.E. Arntz, and R.J. Goris, Signs and symptoms of reflex sympathetic dystrophy: prospective study of 829 patients. Lancet, 1993. 342(8878): p. 1012-6.
4. van de Beek, W.J., R.J. Schwartzman, S.I. van Nes, E.M. Delhaas, and J.J. van Hilten, Diagnostic criteria used in studies of reflex sympathetic dystrophy. Neurology, 2002. 58(4): p. 522-6.
5. Bruehl, S., R.N. Harden, B.S. Galer, S. Saltz, M. Bertram, M. Backonja, R. Gayles, N. Rudin, M.K. Bhugra, and M. Stanton-Hicks, External validation of IASP diagnostic criteria for Complex Regional Pain Syndrome and proposed research diagnostic criteria. International Association for the Study of Pain. Pain, 1999. 81(1-2): p. 147-54.
6. Bruehl, S., R.N. Harden, B.S. Galer, S. Saltz, M. Backonja, and M. Stanton-Hicks, Complex regional pain syndrome: are there distinct subtypes and sequential stages of the syndrome? Pain, 2002. 95(1-2): p. 119-24.
7. Oerlemans, H.M., R.A. Oostendorp, T. de Boo, R.S. Perez, and R.J. Goris, Signs and symptoms in complex regional pain syndrome type I/reflex sympathetic dystrophy: judgment of the physician versus objective measurement. Clin J Pain, 1999. 15(3): p. 224-32.
8. Huygen, F.J., A.G. de Bruijn, J. Klein, and F.J. Zijlstra, Neuroimmune alterations in the complex regional pain syndrome. Eur J Pharmacol, 2001. 429(1-3): p. 101-13.
9. Huygen, F.J., A.G. De Bruijn, M.T. De Bruin, J.G. Groeneweg, J. Klein, and F.J. Zijistra, Evidence for local inflammation in complex regional pain syndrome type 1. Mediators Inflamm, 2002. 11(1): p. 47-51.
10. Huygen, F.J., N. Ramdhani, A. van Toorenenbergen, J. Klein, and F.J. Zijlstra, Mast cells are involved in inflammatory reactions during Complex Regional Pain Syndrome type 1. Immunol Lett, 2004. 91(2-3): p. 147-54.
11. Zijlstra, F.J., I. van den Berg-de Lange, F.J. Huygen, and J. Klein, Anti-inflammatory actions of acupuncture. Mediators Inflamm, 2003. 12(2): p. 59-69.
12. Huygen, F.J., S. Niehof, J. Klein, and F.J. Zijlstra, Computer-assisted skin videothermography is a highly sensitive quality tool in the diagnosis and monitoring of complex regional pain syndrome type I. Eur J Appl Physiol, 2004. 91(5-6): p. 516-24.
13. O'Connor, K.A., A. Holguin, M.K. Hansen, S.F. Maier, and L.R. Watkins, A method for measuring multiple cytokines from small samples. Brain Behav Immun, 2004. 18(3): p. 274-80.
14. Cook, E.B., J.L. Stahl, L. Lowe, R. Chen, E. Morgan, J. Wilson, R. Varro, A. Chan, F.M. Graziano, and N.P. Barney, Simultaneous measurement of six cytokines in a single sample of human tears using microparticle-based flow cytometry: allergics vs. non-allergics. J Immunol Methods, 2001. 254(1-2): p. 109-18.

15. Morgan, E., R. Varro, H. Sepulveda, J.A. Ember, J. Apgar, J. Wilson, L. Lowe, R. Chen, L. Shivraj, A. Agadir, R. Campos, D. Ernst, and A. Gaur, Cytometric bead array: a multiplexed assay platform with applications in various areas of biology. Clin Immunol, 2004. 110(3): p. 252-66.
16. Hodge, G., S. Hodge, R. Haslam, A. McPhee, H. Sepulveda, E. Morgan, I. Nicholson, and H. Zola, Rapid simultaneous measurement of multiple cytokines using 100 microl sample volumes--association with neonatal sepsis. Clin Exp Immunol, 2004. 137(2): p. 402-7.
17. Wrigge, H., U. Uhlig, J. Zinserling, E. Behrends-Callsen, G. Ottersbach, M. Fischer, S. Uhlig, and C. Putensen, The effects of different ventilatory settings on pulmonary and systemic inflammatory responses during major surgery. Anesth Analg, 2004. 98(3): p. 775-81, table of contents.
18. Driessens, M., H. Dijs, G. Verheyen, and P. Blockx, What is reflex sympathetic dystrophy? Acta Orthop Belg, 1999. 65(2): p. 202-17.
19. Huygen, F.J., S. Niehof, F.J. Zijlstra, P.M. van Hagen, and P.L. van Daele, Successful treatment of CRPS 1 with anti-TNF. J Pain Symptom Manage, 2004. 27(2): p. 101-3.
20. Blaha, M., W. Bowers, Jr., J. Kohl, D. DuBose, J. Walker, A. Alkhyyat, and G. Wong, Effects of CEES on inflammatory mediators, heat shock protein 70A, histology and ultrastructure in two skin models. J Appl Toxicol, 2000. 20 Suppl 1: p. S101-8.
21. Schmidt, E., B. Bastian, R. Dummer, H.P. Tony, E.B. Brocker, and D. Zillikens, Detection of elevated levels of IL-4, IL-6, and IL-10 in blister fluid of bullous pemphigoid. Arch Dermatol Res, 1996. 288(7): p. 353-7.
22. Correia, O., L. Delgado, J.C. Roujeau, L. Le Cleach, and J.A. Fleming-Torrinha, Soluble interleukin 2 receptor and interleukin 1alpha in toxic epidermal necrolysis: a comparative analysis of serum and blister fluid samples. Arch Dermatol, 2002. 138(1): p. 29-32.
23. Ying, S., Y. Kikuchi, Q. Meng, A.B. Kay, and A.P. Kaplan, TH1/TH2 cytokines and inflammatory cells in skin biopsy specimens from patients with chronic idiopathic urticaria: comparison with the allergen-induced late-phase cutaneous reaction. J Allergy Clin Immunol, 2002. 109(4): p. 694-700.
24. Gunther, C., G. Wozel, J. Dressler, M. Meurer, and C. Pfeiffer, Tissue eosinophilia in pemphigoid gestationis: association with eotaxin and upregulated activation markers on transmigrated eosinophils. Am J Reprod Immunol, 2004. 51(1): p. 32-9.
25. Wakugawa, M., K. Nakamura, H. Hino, K. Toyama, N. Hattori, H. Okochi, H. Yamada, K. Hirai, K. Tamaki, and M. Furue, Elevated levels of eotaxin and interleukin-5 in blister fluid of bullous pemphigoid: correlation with tissue eosinophilia. Br J Dermatol, 2000. 143(1): p. 112-6.
26. Dadfar, E., J. Lundahl, E. Fernvik, A. Nopp, B. Hylander, and S.H. Jacobson, Leukocyte CD11b and CD62l expression in response to interstitial inflammation in CAPD patients. Perit Dial Int, 2004. 24(1): p. 28-36.
27. Dadfar, E., J. Lundahl, and S.H. Jacobson, Monocyte adhesion molecule expression in interstitial inflammation in patients with renal failure. Nephrol Dial Transplant, 2004. 19(3): p. 614-22.

28. D'Auria, L., P. Cordiali Fei, and F. Ameglio, Cytokines and bullous pemphigoid. Eur Cytokine Netw, 1999. 10(2): p. 123-34.
29. D'Auria, L., M. Pietravalle, P. Cordiali-Fei, and F. Ameglio, Increased tryptase and myeloperoxidase levels in blister fluids of patients with bullous pemphigoid: correlations with cytokines, adhesion molecules and anti-basement membrane zone antibodies. Exp Dermatol, 2000. 9(2): p. 131-7.
30. Fahey, J.V., T.M. Schaefer, J.Y. Channon, and C.R. Wira, Secretion of cytokines and chemokines by polarized human epithelial cells from the female reproductive tract. Hum Reprod, 2005. 20(6): p. 1439-46.
31. Khan, S.S., M.S. Smith, D. Reda, A.F. Suffredini, and J.P. McCoy, Jr., Multiplex bead array assays for detection of soluble cytokines: comparisons of sensitivity and quantitative values among kits from multiple manufacturers. Cytometry B Clin Cytom, 2004. 61(1): p. 35-9.
32. de Jager, W., H. te Velthuis, B.J. Prakken, W. Kuis, and G.T. Rijkers, Simultaneous detection of 15 human cytokines in a single sample of stimulated peripheral blood mononuclear cells. Clin Diagn Lab Immunol, 2003. 10(1): p. 133-9.

Chapter 7

IgE-mediated hypersensitivity: Patients with CRPS type 1 versus the Dutch population. A retrospective study

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Abstract
Objective: To investigate whether hypersensitivity is more common in CRPS1 patients than in the general population. In a recent study, the level of tryptase, a specific marker for mast cells, was significantly higher in blister fluid from the involved extremity of CRPS1 patients. This suggested that mast cells may play a role in the pathophysiology of CRPS1. Mast cells are major effectors in allergic reactions, and are also involved in a variety of non-infectious inflammatory diseases.
Patients: Sixty-six Dutch patients with CRPS1 in one extremity were included.
Outcome measures: Allergy information was obtained from the medical history and a modified questionnaire based on the ELON I study. Total IgE and allergen-specific IgE were measured from blood samples. Also tryptase, as a marker for mast cells, was measured. The data from the questionnaire were compared with that of the general Dutch population, and the plasma levels were compared to reference values and data in the literature.
Results: The medical history did not differ from information provided in the questionnaire by the CRPS1-group. There was no significant difference between the answers to the questionnaire between the CRPS1 patients and the general population. The total IgE levels were elevated in 30% of the CRPS1 patients compared with 15-24% of the general population, and allergen-specific IgE and tryptase levels were comparable with the reference values.
Conclusions: Based on the medical history, an allergy questionnaire, and objective laboratory findings we conclude that IgE-mediated hypersensitivity is not more common in CRPS1 patients than in the general population.

Introduction
Complex Regional Pain Syndrome type 1 (CRPS1) is a disease in the distal part of an extremity that usually occurs as a complication after surgery or trauma, although spontaneous occurrence has also been described (1).
The pathophysiology of CRPS1 remains a matter of debate. In general, three mechanisms are thought to be involved: afferent mechanisms (e.g. neurogenic inflammation), efferent mechanisms (e.g. autonomic disturbances), and central nervous system mechanisms (e.g. cerebral plasticity). Based on our review of the literature regarding the CRPS pathophysiology, we hypothesized that

after trauma or surgery, the normal sterile inflammatory response is exacerbated by a genetic and/or acquired immunologic disorder (2). Neuroimmune activation of cells in the peripheral nervous system, which is part of the afferent mechanism, apparently results in sensitization of the central nervous system, which is clinically displayed as allodynia and hyperalgesia (3). Neuropeptides, cytokines, and other mediators are released during this inflammation (4,5) and cause the classical signs and symptoms, which resemble inflammation. These include increased skin temperature, edema, pain (allodynia, hyperalgesia), loss of function, and redness (2,6).

We showed that the levels of the cytonines tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-6) in fluid from artificially made skin blisters on the involved and contralateral extremities of patients with CRPS1 in one extremity are significantly higher in the involved extremity (4,7). A wide range of cells, such as activated T lymphocytes, monocytes, macrophages, and skin resident cells such as mast cells, could contribute to the production of these cytokines. The involvement of lymphocytes and macrophages is relatively difficult to determine. But the involvement of mast cells is relatively easy to detect by measuring tryptase, a specific marker for mast cells (8).

Mast cells are major effectors in allergic reactions, and are also involved in a variety of non-infectious inflammatory diseases (9). They have many important functions, especially for innate and adaptive immunity, inflammation, and tissue remodelling (10). Mast cells are situated around dermal blood vessels (11) and are in close proximity to nerves in various tissues, including skin, lung and the intestinal mucosa (9,12,13) and a higher number of mast cells have been found in the distal part of extremities (11). Local activated mast cells release vasoactive mediators (histamine, leukotrienes, and prostaglandin D2) and other biologically active molecules (interleukins, proteases and heparin) (14) which causes the wheal-and-flare reaction of the skin (15,16).

Our group measured a significantly higher level of tryptase in blister fluid from the involved extremity which suggested that mast cells may play a role in the production of cytokines such as TNF-α and IL-6 in CRPS1 (17). Because mast cells are also involved in allergic disease, the aim of the study was to investigate whether IgE-mediated hypersensitivity is more common in CRPS1 patients than in a reference population.

Methods

The protocol was approved by the Medical Ethics Committee of the Erasmus MC, Rotterdam (MEC no. 1989.780/2001/24). All patients gave informed consent. The guidelines of the Declaration of Helsinki (amended version of 2002) and Good Clinical Practice (ICH/GCP version 1996) were followed. Data collection and calculations were performed according to guidelines for registration of personal data.

Patients

Sixty-six patients with CRPS1 in one extremity, as defined by the diagnostic criteria of Bruehl (18) (see Table 1), were included in the study. These patients have participated in several studies performed between 2001 and 2005, either to investigate the pathophysiology of CRPS1 or the effects of specific treatment regimens (4,7,17,19).

Registration of allergy

We obtained data on allergy through a medical history taken at the screenings visit to our clinic, prior to the start of medication, between 2001 and 2005. In 2006, all patients received a modified questionnaire based on the ELON I study, a Dutch arm of the European Community Respiratory Health Survey I (ECRHS1) (20,21). Questions about respiratory symptoms concerning asthma, nasal allergies, hay fever, and skin allergies were adopted from the Dutch version of the ECRHS questionnaire.

Those who participated in the ELON 1 study are representative of the Dutch population in general, and were used as a control group. This group was divided into two age groups; a group aged 44 years and younger, and a second group aged 45 years and older. The reason for this was that the first part of the ELON I study was conducted in the 44 years and younger age group, and the investigators wanted to also obtain information about those older than 44 years. The CRPS1 population was also split into similar age groups for comparison.

Plasma

EDTA blood samples (7 ml) were collected from the patients during their screenings visit to our clinic, prior to the start of medication, between 2001 and 2005. In 50 patients with CRPS1 in the upper extremity, blood was obtained by venapuncture in the involved

Table 1. Modified diagnostic criteria for the Complex Regional Pain Syndrome type 1 (18).

Continuing pain which is disproportionate to any inciting event		Report of at least one symptom in each of the following categories	Must display at least one sign in two or more of the following categories
	Sensory:	Hyperesthesia	Evidence of hyperalgesia (to pinprick) and/or allodynia (to light touch)
	Vasomotor:	Temperature asymmetry and/or skin color changes and/or asymmetry	Evidence of temperature asymmetry and/or skin color changes and/or asymmetry
	Sudomotor / oedema:	Oedema and/or sweating changes and/or asymmetry	Evidence of oedema and/or sweating changes and/or asymmetry
	Motor / trophic	Decreased range of motion and/or motor dysfunction (weakness, tremor, dystonia) and/ or trophic changes (hair, nail, skin)	Evidence of decreased range of motion and/or motor dysfunction (weakness, tremor, dystonia) and/or trophic changes (hair, nail, skin)

extremity and a control sample was taken from the contralateral extremity. In 16 patients with CRPS1 in the lower extremity, blood was drawn from the involved extremity, and a control sample was taken from the upper extremity by venapuncture. The plasma was isolated by centrifugation at 2650 gmax for 10 minutes at 20 °C. The samples were stored at -80 °C until analysis.

Laboratory assays

Total IgE, specific IgE against a mixture of inhalant allergens (PhadiaTOP), specific IgE against a mixture of food allergens (fx6 food mix), and tryptase were determined with the ImmunoCAP system (Phadia, Nieuwegein, Netherlands) according to the manufacturers' instructions. The detection limit for total IgE was 2 kU/l; a value of 1 kU/l was given to total serum IgE levels below 2 kU/l. The detection limits for specific IgE and tryptase were 0.35 kU/l it and 1 μg/l, respectively. The Phadia tryptase assay detects both α-tryptase and β-tryptase.

The inhalant allergens mixture (Phadiatop) contained house dust mites, cat, horse, and dog dandruff, grass pollen mixture, mold mixture, tree pollen mixture, herb pollen mixture, and olive tree pollen. The food-allergen mixture consisted of the following allergen groups: chicken egg whites, cow's milk, codfish, wheat, peanut, and soy.

The levels of C-reactive protein (CRP) were measured with a high-sensitivity assay (hs-CRP) with the Immage 800 Immunochemistry System (Beckman Coulter, Mijdrecht, The Netherlands). The assay is calibrated against the CAP (College of American Pathologists) standard for CRPH, with a range from 0.2-1440 mg/l. All measurements were performed in the laboratories of the department of clinical chemistry of the Erasmus Medical Center, Rotterdam, The Netherlands.

Statistical analysis

Data were analyzed with SPSS for Windows, version 14.0. For analysis of the questionnaire, the cross-tabs were calculated and the results were analyzed with the Chi-square test. A p-value $\leq$ 0.05 divided by the number of comparisons (13 questions) was considered to indicate a significant difference (a Bonferroni correction). Correlation of paired samples was calculated for laboratory analysis using Spearman's Rank Correlation test. A p-value $\leq$ 0.05 was considered to indicate a significant correlation.

Results

A group of 66 patients with CRPS1 in one extremity, with no systemic inflammation involved (normal levels of CRP in plasma), was included in this study. The characteristics of the patients are presented in Table 2.

Table 2. Characteristics of the study population.

Patient characteristic	Value
Gender: male / female	16 / 50
Age in years	50 (21 – 80)
Duration of CRPS1, months	22 (2 – 193)
Location: upper / lower extremity	50 / 16
Side: right / left	34 / 32
Cause: trauma (fracture, accident) / surgery / spontaneous / other	40 (25, 15) / 17 / 2 / 7
Plasma CRP: involved side / contralateral side (mg/l)	2.8 (0.2 – 39.4) / 2.6 (0.2 – 43.7)

Data are presented as median (range)

Thirty-three of the 62 patients reported an allergy in their medical history. No information about allergies was available for 4 patients. An allergy to antibiotics was reported by 24.2%, to NSAIDs by 9%, and for corticosteroids and calcium antagonists by 3% each. A skin reaction, mainly caused by plasters and metals, was reported by 39.4% of these patients and 6% reported an allergy to insect bites. An allergy caused mainly by pollen and house dust mites was reported by 24.2% of the patients, and a food allergy was reported by 6%.

The modified questionnaire was sent to the 66 CRPS1 patients, but 11 were not returned. No difference was found in the CRPS1 group between the answers given on the medical history and the answers given on the questionnaire. Based on the Chi-square test, no significant difference in answers between the CRPS1 population and the controls in both age groups was found (Table 3).

Plasma was available from 63 of the 66 CRPS1 patients. A normal level of total serum IgE (<100 kU/L) was found in the plasma of 70% of the 63 CRPS1 patients (range; 2-83 kU/L). There was an increase in the IgE level (>100 kU/L) in the other 30% of the patients (range; 109-1900 kU/L). The measurements for allergen-specific IgE were performed for aeroallergens and for food

Table 3. Allergy questionnaire: CRPS1 patients and the Dutch population.

	CRPS1 patients (N=20) ≤ 44 years (% yes)	Dutch population (N=1314) ≤ 44 years (% yes)	p-value	CRPS1 patients (N=35) ≥ 45 years (% yes)	Dutch population (N=1397) ≥ 45 years (% yes)	p-value
Nasal allergy including hay fever?	35.0	18.7	0.081	23.5	14.1	0.134
Wheezing or whistling in your chest?	10.0	20.4	0.399	25.7	26.9	1.000
If yes, where you short of breath?	30.0	14.3	0.058	22.9	17.1	0.366
If yes, you did not have a cold?	15.0	12.2	0.727	23.5	13.8	0.129
Woken up with a feeling of tightness in your chest?	5.0	8.8	1.000	15.6	15.3	0.367
Woken up by an attack of shortness of breath?	15.0	4.9	0.075	6.3	7.8	1.000
Woken up by an attack of coughing?	40.0	30.3	0.338	15.6	33.4	0.036
Medication taken for asthma?	10.0	2.1	0.072	8.6	2.9	0.089
Trees, grass, flowers or a lot of pollen in the air:						
Get runny or stuffy nose?	35.0	18.1	0.074	26.5	14.7	0.083
Get itchy or watering eyes?	35.0	14.4	0.019	17.6	10.5	0.168
Feathers or dust:						
Get runny or stuffy nose?	42.1	27.2	0.192	20.0	16.4	0.497
Get itchy or watering eyes?	15.0	14.7	1.000	8.6	9.8	1.000
Eczema or any kind of skin allergy?	57.9	36.2	0.058	28.6	29.9	1.000

Chi-square test p-value < 0.05/13 (Bonferroni correction)

allergens. In 62 patients (94%), no IgE against food allergens was found (<0.35 kU/L). In 68% of the patients, no IgE against inhalant allergens was found (<0.35 kU/L). In the other 32% of patients, the levels of aeroallergen-specific IgE were elevated compared with the reference value of <0.35 kU/L, with a range of 0.36-27.6 kU/L. The levels of tryptase were normal (<11.4 μg/l) in 91% of the patients (range; 2.0-10.2 μg/l), but were elevated in 9% of the patients (range; 11.5-19.6 μg/l).

Discussion

In this study, similar answers were given on the medical history and on the questionnaire based on the ELON I study in the CRPS1 population. We also did not find any significant difference between the CRPS1 population and the general population. In the plasma of the CRPS1 patients, only a slight increase in tryptase was found. Total IgE was increased in 30% of the patients, but the levels of aeroallergen-specific IgE and food allergen-specific IgE were normal compared with the reference values.

Allergic asthma, allergic rhinitis (hay fever), or atopic eczema are frequent conditions and affect at least 20% and up to 40% of the adult individuals in Western societies (22-24). Allergic rhinitis is the most frequent condition. In recent decades, it is suggested that the prevalence of these conditions has increased (23,25). The ELON I study was performed in 1992, and our patients filled out the questionnaire in 2006. If the suggestion of increased atopy during the last decade is true, repetition of the ELON I study would give a higher prevalence of symptoms at the present time. One possibility would be that the percentages from the CRPS1 population would be even lower than in the general Dutch population instead of the same.

In the pathogenesis of allergic reaction, mast cells are involved in some inflammatory skin reactions (16) and in inflammatory reactions in CRPS1 patients (17). When mast cells are activated by specific antigens, surface-bound antigen-specific IgE (22,26,27) or stress local degranulation of the secretory granules may occur (28). This can cause release of vasoactive mediators and other biologically active molecules (14) and may induce the wheal-and-flare reaction of the skin (15,16). This local reaction may play a role in CRPS1 and may cause the redness and swelling in the acute stage of the disease. In the plasma of CRPS1 patients, an increase in IL-8 was found by Schinkel et al., but no increase in

IL-6 was found (29). Van de Beek et al. could not find any increase in cytokines in the plasma of CRPS1 patients (30). However, in the blister fluid of patients with CRPS1, the interleukins TNF-α and IL-6 are increased in the involved extremity (4,31). This may indicate a local process with no systemic activation of these cells.

Tryptase is a good marker for systemic mast cell activation (10,27,32). Min et al. found that the levels of total tryptase are higher in men than in women (33). But there are several diseases, for example Multiple Sclerosis, which occur more often in women than in men and the levels of tryptase and histamine are found to be higher in the cerebral spinal fluid. CRPS1 also occurs more often in women than in men (34). Huygen et al found significantly higher levels of tryptase in suction blister fluid from the involved extremity of CRPS1 patients (17), but we could not find significantly increased levels of tryptase in the plasma from our patients. This may indicate a local role for mast cells in CRPS1 rather than a systemic one.

IgE-mediated hypersensitivity consists of two stages. The initial sensitization phase occurs following exposure of a predisposed but IgE antibody-negative individual to an allergenic substance; for example, house dust mites. This will lead to production of specific IgE antibodies. IgE circulates in the blood and binds to high affinity epsilon Fc-receptors on mast cells and basophils. In the second phase, the sensitized individual may experience any of a spectrum of allergy symptoms upon re-exposure to the same allergen. These symptoms can range from asthma, urticaria, or anaphylactic shock. Only 50% of those sensitized to allergens express these symptoms (35). IgE can be increased in all kinds of diseases, such as in atopy, infection, immunological disorders or parasitic disease, and also in people without disease. This means that total IgE has a very limited diagnostic value for any condition. It is only a good indicator of the prevalence of sensitization to allergens in the population and to decide if further research is necessary (22,27,36,37). Because of the presence of increased total IgE in many diseases, we measured IgE in the plasma of CRPS1 patients. We found an elevated level of IgE in 30% of the CRPS1 patients, which is higher than the 15-24% reported in the literature (38-40). A possible sensitization to allergens might be on hand, and to confirm or exclude this it is necessary to perform specific IgE tests to common aeroallergens and food allergens.

Hypersensitivity to aeroallergens and food hypersensitivity are both based on a IgE-dependent mechanism, and mast cells and cytokines are the things involved in this mechanism (15) as well as in CRPS1 (4,17). The next research step for us in CRPS was to investigate whether patients with CRPS1 are hypersensitive to aeroallergens and food allergens. It is important to first look at the clinical history of the patient to diagnose hypersensitivity to aeroallergens and food allergens. The diagnosis can be further established with either skin-prick tests or measurement of specific IgE (23,27). The skin-prick test was not possible in the CRPS1 population because we were no longer seeing the patients in our clinic. We were only able to measure the aeroallergen-specific and food allergen-specific IgE in the plasma we had in stock from this population.

At least one positive specific IgE test to common inhalant aeroallergens is seen as the golden standard for sensitization (36). In 32% of the general Dutch population, Kerkhof et al. found at least one positive allergen-specific IgE test in both men and women (41). This is exactly the same percentage we found in our CRPS1 population. This means that the level of allergen-specific IgE in CSPS1 patients does not deviate from the general Dutch population. Therefore, we conclude that CRPS1 patients do not have a higher prevalence of hypersensitivity to aeroallergens.

The prevalence of food hypersensitivity in adults is about 3%, and has increased in recent decades in the general adult population. But there is a large discrepancy between the self-reported prevalence and the confirmed prevalence by food challenge (10-14% vs. 3-3.2%)(15,42,43). In our population 6% reported a food allergy during the medical history, but a slight increase in food specific IgE was found in only 1 patient (1.6% of the total group). These percentages are lower than those in the general population, which means that our population does not have hypersensitivity to food.

In summary, based on the medical history, an allergy questionnaire, and objective laboratory findings we conclude that IgE-mediated hypersensitivity for aeroallergens and for food allergens is not more common in CRPS1 patients than in the general Dutch population.

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References

1. Janig W, Baron R. Complex regional pain syndrome: mystery explained? Lancet Neurol 2003;2:687-697.
2. Huygen FJ, de Bruijn AG, Klein J, Zijlstra FJ. Neuroimmune alterations in the complex regional pain syndrome. Eur J Pharmacol 2001;429:101-113.
3. Alexander GM, van Rijn MA, van Hilten JJ, Perreault MJ, Schwartzman RJ. Changes in cerebrospinal fluid levels of pro-inflammatory cytokines in CRPS. Pain 2005;116:213-219.
4. Huygen FJ, De Bruijn AG, De Bruin MT, Groeneweg JG, Klein J, Zijistra FJ. Evidence for local inflammation in complex regional pain syndrome type 1. Mediators Inflamm 2002;11:47-51.
5. Birklein F. Complex regional pain syndrome. J Neurol 2005; 252:131-138.
6. Schwartzman RJ, Popescu A. Reflex sympathetic dystrophy. Curr Rheumatol Rep 2002;4:165-169.
7. Huygen FJ, Niehof S, Zijlstra FJ, van Hagen PM, van Daele PL. Successful treatment of CRPS 1 with anti-TNF. J Pain Symptom Manage 2004;27:101-103.
8. Deleuran B, Kristensen M, Larsen CG, Matsson P, Enander I, Andersson AS, Thestrup-Pedersen K. Increased tryptase levels in suction-blister fluid from patients with urticaria. Br J Dermatol 1991;125:14-17.
9. Ito A, Oonuma J. Direct interaction between nerves and mast cells mediated by the SgIGSF/SynCAM adhesion molecule. J Pharmacol Sci 2006;102:1-5.
10. Rueff F, Placzek M, Przybilla B. Mastocytosis and Hymenoptera venom allergy. Curr Opin Allergy Clin Immunol 2006;6:284-288.
11. Janssens AS, Heide R, den Hollander JC, Mulder PG, Tank B, Oranje AP. Mast cell distribution in normal adult skin. J Clin Pathol 2005;58:285-289.
12. Bauer O, Razin E. Mast Cell-Nerve Interactions. News Physiol Sci 2000;15:213-218.
13. Gurish MF, Boyce JA. Mast cells: ontogeny, homing, and recruitment of a unique innate effector cell. J Allergy Clin Immunol 2006;117:1285-1291.
14. van Toorenenbergen AW, Oranje AP. Comparison of serum tryptase and urine N-methylhistamine in patients with suspected mastocytosis. Clin Chim Acta 2005;359:72-77.
15. Moneret-Vautrin AD. Gastrointestinal allergy in adults. Eur J Gastroenterol Hepatol 2005;17:1293-1297.
16. Amon U, Menz U, Wolff HH. Investigations on plasma levels of mast cell mediators in acute atopic dermatitis. J Dermatol Sci 1994;7:63-67.
17. Huygen FJ, Ramdhani N, van Toorenenbergen A, Klein J, Zijlstra FJ. Mast cells are involved in inflammatory reactions during Complex Regional Pain Syndrome type 1. Immunol Lett 2004;91:147-154.
18. Harden RN, Bruehl S, Galer BS, Saltz S, Bertram M, Backonja M, Gayles R, Rudin N, Bhugra MK, Stanton-Hicks M. Complex regional pain syndrome: are the IASP diagnostic criteria valid and sufficiently comprehensive? Pain 1999;83:211-219.
19. Munnikes RJ, Muis C, Boersma M, Heijmans-Antonissen C, Zijlstra FJ, Huygen FJ. Intermediate stage complex regional pain syndrome type 1 is unrelated to proinflammatory cytokines. Mediators Inflamm 2005:366-372.

20. Kerkhof M, de Graaf A, Droste J, Cardynaals R, de Monchy J, Rijcken B: The prevalence of asthma-like symptoms in three areas in the Netherlands. In Tijdschrift Sociale Gezondheidszorg. 1994:181-185.
21. Rijcken B, Kerkhof M, de Graaf A, Boezen H, Droste J, Kremer A: Europees Luchtweg Onderzoek Nederland (ELON). Groningen, Rijksuniversiteit Groningen, 1996.
22. Winter WE, Hardt NS, Fuhrman S. Immunoglobulin E: importance in parasitic infections and hypersensitivity responses. Arch Pathol Lab Med 2000;124:1382-1385.
23. Hilliquin P, Allanore Y, Coste J, Renoux M, Kahan A, Menkes CJ. Reduced incidence and prevalence of atopy in rheumatoid arthritis. Results of a case-control study. Rheumatology (Oxford) 2000;39:1020-1026.
24. Variations in the prevalence of respiratory symptoms, self-reported asthma attacks, and use of asthma medication in the European Community Respiratory Health Survey (ECRHS). Eur Respir J 1996;9:687-695.
25. Jarvis D, Luczynska C, Chinn S, Potts J, Sunyer J, Janson C, Svanes C, Kunzli N, Leynaert B, Heinrich J, Kerkhof M, Ackermann-Liebrich U, Anto JM, Cerveri I, de Marco R, Gislason T, Neukirch F, Vermeire P, Wjst M, Burney P. Change in prevalence of IgE sensitization and mean total IgE with age and cohort. J Allergy Clin Immunol 2005;116:675-682.
26. Metcalfe DD, Baram D, Mekori YA. Mast cells. Physiol Rev 1997;77:1033-1079.
27. Hamilton RG, Adkinson NF, Jr. 23. Clinical laboratory assessment of IgE-dependent hypersensitivity. J Allergy Clin Immunol 2003;111:S687-701.
28. Theoharides TC, Donelan J, Kandere-Grzybowska K, Konstantinidou A. The role of mast cells in migraine pathophysiology. Brain Res Brain Res Rev 2005;49:65-76.
29. Schinkel C, Gaertner A, Zaspel J, Zedler S, Faist E, Schuermann M. Inflammatory mediators are altered in the acute phase of posttraumatic complex regional pain syndrome. Clin J Pain 2006;22:235-239.
30. van de Beek WJ, Remarque EJ, Westendorp RG, van Hilten JJ. Innate cytokine profile in patients with complex regional pain syndrome is normal. Pain 2001;91:259-261.
31. Heijmans-Antonissen C, Wesseldijk F, Munnikes RJ, Huygen FJ, van der Meijden P, Hop WC, Hooijkaas H, Zijlstra FJ. Multiplex bead array assay for detection of 25 soluble cytokines in blister fluid of patients with complex regional pain syndrome type 1. Mediators Inflamm 2006:28398.
32. Akin C, Metcalfe DD. Surrogate markers of disease in mastocytosis. Int Arch Allergy Immunol 2002;127:133-136.
33. Min HK, Moxley G, Neale MC, Schwartz LB. Effect of sex and haplotype on plasma tryptase levels in healthy adults. J Allergy Clin Immunol 2004;114:48-51.
34. de Mos M, de Bruijn AG, Huygen FJ, Dieleman JP, Stricker BH, Sturkenboom MC. The incidence of complex regional pain syndrome: a population-based study. Pain 2007;129:12-20.
35. Hamilton RG. Laboratory analyses in the diagnosis of human allergic disease. Methods 1997;13:25-32.
36. Kerkhof M, Dubois AE, Postma DS, Schouten JP, de Monchy JG. Role and interpretation of

total serum IgE measurements in the diagnosis of allergic airway disease in adults. Allergy 2003;58:905-911.
37. Barbee RA, Halonen M, Lebowitz M, Burrows B. Distribution of IgE in a community population sample: correlations with age, sex, and allergen skin test reactivity. J Allergy Clin Immunol 1981;68:106-111.
38. Wittig HJ, Belloit J, De Fillippi I, Royal G. Age-related serum immunoglobulin E levels in healthy subjects and in patients with allergic disease. J Allergy Clin Immunol 1980;66:305-313.
39. Sears MR, Chow CM, Morseth DJ. Serum total IgE in normal subjects and the influence of a family history of allergy. Clin Allergy 1980;10:423-431.
40. Carosso A, Bugiani M, Migliore E, Anto JM, DeMarco R. Reference values of total serum IgE and their significance in the diagnosis of allergy in young European adults. Int Arch Allergy Immunol 2007;142:230-238.
41. Kerkhof M, Droste JH, de Monchy JG, Schouten JP, Rijcken B. Distribution of total serum IgE and specific IgE to common aeroallergens by sex and age, and their relationship to each other in a random sample of the Dutch general population aged 20-70 years. Dutch ECRHS Group, European Community Respiratory Health Study. Allergy 1996;51:770-776.
42. Osterballe M, Hansen TK, Mortz CG, Host A, Bindslev-Jensen C. The prevalence of food hypersensitivity in an unselected population of children and adults. Pediatr Allergy Immunol 2005;16:567-573.
43. Jansen JJ, Kardinaal AF, Huijbers G, Vlieg-Boerstra BJ, Martens BP, Ockhuizen T. Prevalence of food allergy and intolerance in the adult Dutch population. J Allergy Clin Immunol 1994;93:446-456.

General discussion

General discussion

Our hypothesis was that an inflammatory process would be present in the initial stage of CRPS1. We based this hypothesis on the clinical picture of CRPS1, which presents with signs and symptoms resembling features of inflammation, including pain, heat, edema, and decreased mobility. A variety of mediators of inflammation could be responsible for this inflammatory process and subsequently cause the clinical signs and symptoms commonly seen in CRPS1. Furthermore, we hypothesized that the inflammatory process would diminish over time by normalization of the mediators of inflammation. This hypothesis was based on changes in clinical signs and symptoms seen in CRPS1 throughout the course of the disease.

To test our hypothesis of an inflammatory reaction in CRPS1, we measured proinflammatory cytokines. As the most commonly identified cytokines, TNF-α and IL-6 were the most likely initial candidates. In previous studies, these mediators were measured in plasma, but no differences in cytokine levels were observed between CRPS1 patients and controls [1, 2]. Considering the fact that the signs and symptoms generally present locally in an extremity, we concluded that the cytokines should be measured in a more local sample of tissue fluid. Using a suction method, we artificially induced skin blisters in the affected and contralateral extremities of study participants and measured the levels of TNF-α and IL-6 in the blister fluid. We found that TNF-α and IL-6 levels were increased in the CRPS1 side compared to the contralateral extremity in the first 2–3 years of the disease (Chapters 2 and 3, Figure 1) [3, 4]. These findings are in agreement with earlier results found in CRPS1 patients [1, 5].

A remarkable finding from our work with TNF-α and IL-6 is the fact that about half of the patients showed no signs of an inflammatory process, such as increased temperature and edema [6, 7]. Six years after the initial event, differences in levels of TNF-α and IL-6 between the CRPS1 side and the contralateral extremity had diminished. However, some patients still showed signs and symptoms of inflammation [7]. Furthermore, in two other studies, a correlation between levels of proinflammatory cytokines and the disease characteristics pain, and changes in temperature, volume and mobility, and disease duration was lacking [6, 7]. Although there was a decrease of in levels of proinflammatory cytokines in our study, which confirms our hypothesis, the decrease did not

correspond with the signs and symptoms of inflammation seen in the CRPS1 patients. These findings contradict our hypothesis. We can thus conclude that TNF-α and IL-6 are involved in CRPS1 but are most probably individually not responsible for the clinical features of CRPS1.

The suction method to induce blisters may in itself be a trigger for mediators to be released or produced in the involved (damaged) skin, which could explain the increased levels of cytokines in the CRPS1 extremity compared to the contralateral side. However, 6 years after the initial event, the levels were similar in the CRPS1 side compared to the contralateral extremity. A change in skin structure over time might be a reason that the vacuum of negative pressure could not trigger the production or release of the mediators at that stage. Another reason for changes in the levels of the mediators might be changes in permeability to these mediators in the skin and/or the blood vessels during the course of the disease.

The location of the blister could have varied from the location of peak symptoms, a possible explanation for why we did not find a correlation between the levels of TNF-α and IL-6. Patients do not always exhibit a generalized pattern of signs and symptoms. Videothermography shows that patients have a patchy pattern of the skin temperature of the extremity, and one part of the hand or foot may be more affected by the disease than another part.

A cluster analysis, as done by Bruehl et al., could possibly identify a clearer correlation between TNF-α and IL-6 levels and the signs and symptoms of CRPS1. The most likely subgroup would be the one that exhibits the most florid overall CRPS syndrome [8]. We could not perform such an analysis because the number of patients examined was too small.

Thus, the inflammatory mediators TNF-α and IL-6 have been suggested as potential contributors to CRPS1, but these proinflammatory cytokines do not correlate with the clinical manifestations of the syndrome. For this reason, we investigated the role of amino acids in the pathogenesis of CRPS1, focusing especially on amino acids related to the NMDA receptor and nitric oxide synthesis. As Chapter 4 describes, relative to controls, plasma levels in CRPS1 patients of glutamate, arginine, taurine, and glycine were increased, and plasma levels of glutamine were decreased (Figure 1). However, the subjective measures of pain and indicators of psycho neuroticism and emotional instability

did not correlate with amino acid levels [9]. Chapter 5 reports on our investigation into the role of plasma 5-HT in CRPS1, which identified significantly elevated levels of plasma 5-HT in CRPS1 patients compared to controls (Figure 1). Again, however, we also found a lack of correlation with distinct disease characteristics [10]. We can conclude that, like TNF-α and IL-6, the examined amino acids are individually not responsible for the complete picture of signs and symptoms of CRPS1.

It is possible that mediators of inflammation from the categories of neuropeptides and eicosanoids, which we did not measure in this set of investigations, are elevated in CRPS1 patients. These mediators also can cause the pain, temperature changes, and edema seen in CRPS1 (Figure 1). In a previous study, increased levels of serum CGRP in CRPS patients were correlated with the incidence of nerve lesions and hyperhidrosis. There was no correlation with other clinical symptoms, duration of CRPS, or pain. However, normalization of CGRP after therapy was accompanied by clinical improvement of local inflammatory signs but not by pain reduction [11]. In the future, it may be worthwhile to investigate whether mediators from the abovementioned categories are changed in CRPS1 patients and whether they correlate with the signs and symptoms of the disease measured in these patients.

Based on a review of the literature on pathophysiology in CRPS [12], Huygen et al. hypothesized that, as the result of a genetic and/or acquired immunologic disorder, a normal sterile inflammatory response after trauma or surgical procedure becomes exacerbated. Secondarily, based on sensitization of the dorsal horn and higher centers, sensory, vasomotor, sudomotor, motor, and trophic changes develop, which also could be responsible for some of the changes in signs and symptoms during the course of the disease [5]. A combination of these afferent, efferent, and central mechanisms can demonstrate that the signs and symptoms seen in CRPS1 are different, as would be expected as an effect of an inflammatory reaction. Still, there is the issue of whether there is a sympathetic component together with an inflammatory component and whether the sympathetic nervous system contributes to the early inflammatory state. Furthermore, it is not clear whether the alterations in the central nervous system are primary abnormalities in CRPS1 or whether they are changes secondary to pain [13]. If only the afferent mechanism is activated in the initial stage of the disease, a correlation between the levels

of the proinflammatory mediators and the signs and symptoms would be expected. However, this has not been found in the studies reported in this thesis [6, 7].
The initial trauma could also elicit release of anti-inflammatory mediators, such as IL-10, which also could suppress the effects of the proinflammatory mediators. Such a mechanism would explain why the clinical features of CRPS1 are not the same in the different stages of the disease, as we had initially expected.
Another possible explanation for these findings is that there are as-yet-unknown mediators or cells involved in the complex mechanism that produces the signs and symptoms of CRPS1. In addition, current measurement techniques may not detect these mediators or cells. However, we used new measurement techniques in the study described in Chapter 6. The use of a multiplex-25 bead array cytokine assay and LuminexTM technology enabled simultaneous measurement of representative pro-inflammatory cytokines, Th1/Th2 distinguishing cytokines, non-specifically acting cytokines and chemokines. Ten representative cytokines were detectable; in addition to IL-6 and TNF-α, these included IL-1RA, IL-8, IL-12, MCP-1, and MIP-1β, all detectable and all increased in CRPS1 affected extremities. However, IP-10 and eotaxin were detectable but diminished in CRPS1 [14]. In the future, it could be worthwhile to measure these mediators in blister fluid of CRPS1 patients again and correlate them with the signs and symptoms of the disease. One or more of these mediators might be responsible for the clinical manifestations of the disease.
We also know that these mediators are probably released from monocytes, macrophages and mast cells. Tryptase is a specific marker for mast cells and was found to be increased in blister fluid from the CRPS1 extremity; thus, mast cells could play a role in the production of cytokines in CRPS1 [5]. Mast cells are also known to be involved in allergic disease. Another explanation for the initiation or predisposition of CRPS1 could be that hypersensitivity plays a role. In Chapter 7 we investigated this hypothesis and found that based on the medical history of the CRPS1 patients, responses to an allergy questionnaire, and objective laboratory findings, such as IgE and tryptase, hypersensitivity for aeroallergens and food allergens is not more common in CRPS1 patients than in the general Dutch population [15]. We conclude that hypersensitivity/allergic disease does not play a role in the

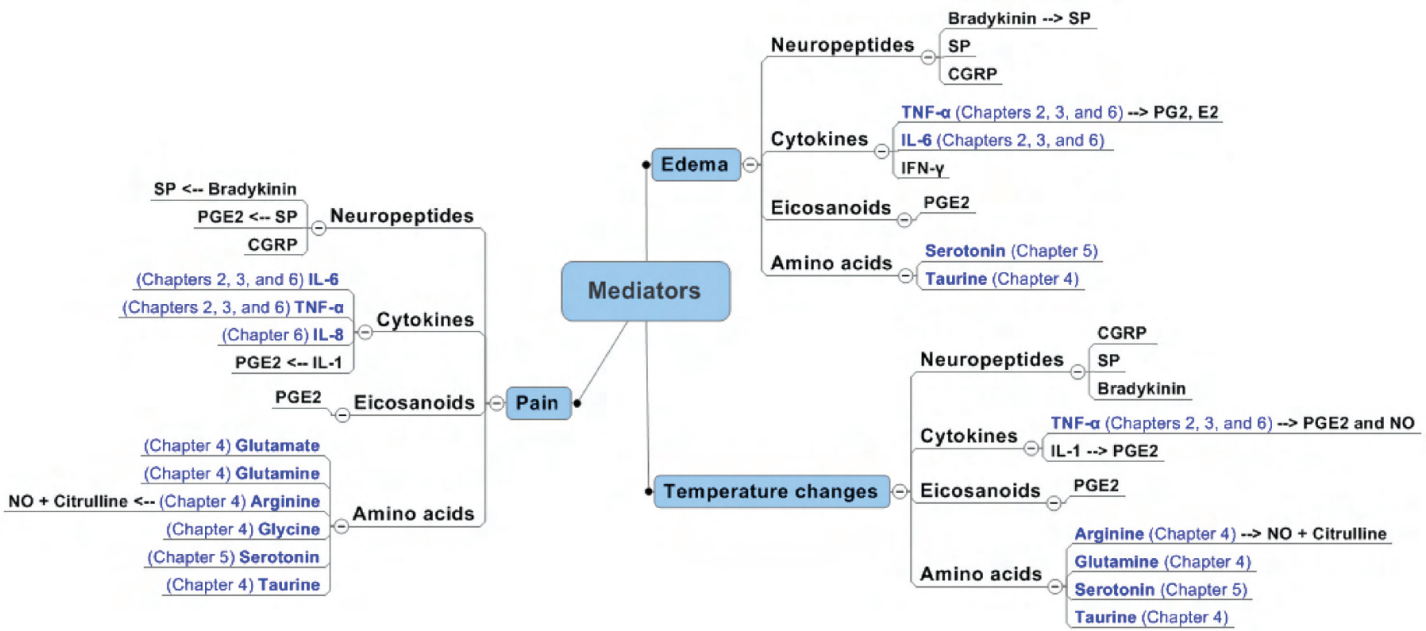

Figure 1. Mediators that are reviewed in the Introduction. All the mediators that are significantly changed in CRPS1 patients measured in this thesis are marked in blue.
SP, substance P; CGRP, calcitonin-gene related peptide; TNF-α, tumor necrosis factor alpha; IL-1, interleukin-1; IL-6, interleukin-6; IL-8, interleukin-8 ; PGE2, prostaglandin E2; NO, nitric oxide.

initiation or predisposition of CRPS1. In Chapter 6 we also report our findings, through a different approach, that allergic reactions do not play an important role in CRPS1. Eotaxin and IL-5 are representative chemotactic cytokines for studying the activation of skin-homed eosinophils, which in general represent allergic reactions. We found that IL-5 was not detectable and eotaxin was surprisingly decreased in CRPS1 blister fluid.
There also might be mediators or cells that we can measure but that are not known to cause an inflammatory reaction with pain, allodynia, edema, and/or temperature changes. They could have their own place and function in the complex mechanism, as well. Finally, the mediators measured in CRPS1 patients in this thesis were individually not responsible for the characteristics of CRPS1, but a combination of these mediators might be responsible for, among other things, the pain, edema, and temperature changes seen in CRPS1.

Conclusion

In this thesis, we found that a variety of mediators of inflammation are increased in tissue fluid of CRPS1 patients. In blister fluid, we found increased levels of TNF-α and IL-6. These findings are in agreement with earlier results from studies involving CRPS1 patients [1, 5]. In plasma, we identified increased levels of glutamate, arginine, taurine, glycine, and serotonin and decreased levels of glutamine (Figure 1). However, these individually could not explain the clinical signs and symptoms seen in CRPS1, which resemble the characteristics of inflammation, and also were not correlated with the duration of the disease (Chapters 2–5).
Levels of a couple of mediators are also changed in CRPS1 patients, but whether they are responsible for the clinical manifestation of the disease remains unknown (Chapter 6). Furthermore, IgE-mediated hypersensitivity is not an explanation for CRPS1 (Chapter 7). We conclude that these known mediators, possibly also with as-yet-unknown mediators, cells and/or mechanisms, or a combination of these are a potential explanation for the initiation, continuation and/or changes in clinical signs and symptoms of CRPS1 during the course of the disease.

References

1. Huygen, F.J., A.G. De Bruijn, M.T. De Bruin, J.G. Groeneweg, J. Klein, and F.J. Zijistra, Evidence for local inflammation in complex regional pain syndrome type 1. Mediators Inflamm, 2002. 11(1): p. 47-51.
2. van de Beek, W.J., E.J. Remarque, R.G. Westendorp, and J.J. van Hilten, Innate cytokine profile in patients with complex regional pain syndrome is normal. Pain, 2001. 91(3): p. 259-61.
3. Wesseldijk, F., F.J. Huygen, C. Heijmans-Antonissen, S.P. Niehof, and F.J. Zijlstra, Tumor necrosis factor-alpha and interleukin-6 are not correlated with the characteristics of Complex Regional Pain Syndrome type 1 in 66 patients. Eur J Pain, 2008. 12(6): p. 716-21.
4. Wesseldijk, F., F.J. Huygen, C. Heijmans-Antonissen, S.P. Niehof, and F.J. Zijlstra, Six years follow-up of the levels of TNF-alpha and IL-6 in patients with complex regional pain syndrome type 1. Mediators Inflamm, 2008. 2008: p. 469439.
5. Huygen, F.J., N. Ramdhani, A. van Toorenenbergen, J. Klein, and F.J. Zijlstra, Mast cells are involved in inflammatory reactions during Complex Regional Pain Syndrome type 1. Immunol Lett, 2004. 91(2-3): p. 147-54.
6. Wesseldijk, F., F.J. Huygen, C. Heijmans-Antonissen, S. Niehof, and F.J. Zijlstra, Tumor necrosis factor-α and interleukin-6 are not correlated with the characteristics of complex regional pain syndrome type 1 in 66 patients. Eur J Pain, 2007. doi:10.1016/j.ejpain.2007.10.010.
7. Wesseldijk, F., F.J. Huygen, C. Heijmans-Antonissen, S. Niehof, and F.J. Zijlstra, Six years follow-up of the levels of TNF-α and IL-6 in patients with complex regional pain syndrome type 1. Mediators Inflamm, 2008. Accepted for Publication.
8. Bruehl, S., R.N. Harden, B.S. Galer, S. Saltz, M. Backonja, and M. Stanton-Hicks, Complex regional pain syndrome: are there distinct subtypes and sequential stages of the syndrome? Pain, 2002. 95(1-2): p. 119-24.
9. Wesseldijk, F., D. Fekkes, F.J. Huygen, M. van de Heide-Mulder, and F.J. Zijlstra, Increased plasma glutamate, glycine, and arginine levels in complex regional pain syndrome type 1. Acta Anaesthesiol Scand, 2008. 52(5): p. 688-94.
10. Wesseldijk, F., D. Fekkes, F.J. Huygen, E. Bogaerts-Taal, and F.J. Zijlstra, Increased plasma serotonin in complex regional pain syndrome type 1. Anesth Analg, 2008. 106(6): p. 1862-7.
11. Birklein, F., M. Schmelz, S. Schifter, and M. Weber, The important role of neuropeptides in complex regional pain syndrome. Neurology, 2001. 57(12): p. 2179-84.
12. Huygen, F.J., A.G. de Bruijn, J. Klein, and F.J. Zijlstra, Neuroimmune alterations in the complex regional pain syndrome. Eur J Pharmacol, 2001. 429(1-3): p. 101-13.
13. Wasner, G., J. Schattschneider, A. Binder, and R. Baron, Complex regional pain syndrome--diagnostic, mechanisms, CNS involvement and therapy. Spinal Cord, 2003. 41(2): p. 61-75.
14. Heijmans-Antonissen, C., F. Wesseldijk, R.J. Munnikes, F.J. Huygen, P. van der Meijden, W.C. Hop, H. Hooijkaas, and F.J. Zijlstra, Multiplex bead array assay for detection of 25 soluble cytokines in blister fluid of patients with complex regional pain syndrome type 1. Mediators Inflamm, 2006(1): p. 28398.
15. Wesseldijk, F., A. van Toorenenbergen, R. Gerth van Wijk, F.J. Huygen, and F.J. Zijlstra,

IgE-mediated hypersensitivity: Patients with Complex Regional Pain Syndrome type 1 versus the Dutch population. A retrospective study Pain Med, May, 2008. Resubmitted

Summary

Summary
Complex regional pain syndrome type 1 (CRPS1) is a disease of the extremity that usually occurs as a complication after surgery or trauma, although spontaneous occurrence has also been described. The prominent signs and symptoms seen in the initial stage of CRPS resemble the characteristics of inflammation, such as pain, increased temperature and color changes of the skin, swelling of the extremity, and loss of function. We hypothesized that a variety of mediators of inflammation should be present in the tissue fluid of patients with CRPS1 and could be responsible for this inflammatory process, subsequently causing the clinical signs and symptoms commonly seen in CRPS1. Furthermore, the inflammatory process would be expected to diminish over time by normalization of the mediators of inflammation. These ideas were based on the clinical signs and symptoms seen in CRPS1 during the course of the disease.
An earlier study demonstrated that the proinflammatory cytokines TNF-α and IL-6 remained unchanged in plasma from CRPS1 patients. Because of this finding and because the signs and symptoms present locally in an extremity, these two mediators were measured in a more local sample of tissue fluid. Chapter 2 describes measurement of the levels of TNF-α and IL-6 in artificially induced blister fluid. Of the 66 CRPS1 patients assessed in the first 2 years of the disease, the levels were significantly increased on the CRPS1 side compared to the contralateral extremity, but only about half of the patients showed signs of inflammation. No correlation could be found between the cytokine and the characteristic pain, changes in temperature, volume and mobility or duration of the disease.
Chapter 3 describes findings determined 6 years after the initial event, showing that TNF-α and IL-6 levels had diminished in the CRPS1 side of 12 patients of the original 66. However, pain, temperature, and volume did not change from their values at the initial stage, and only mobility improved after 6 years. Signs of inflammation were still present at this stage of the disease. Again, no correlation could be found between cytokine levels and the signs and symptoms of CRPS1 during the course of the disease. From the findings of these two studies, we concluded that the proinflammatory cytokines TNF-α and IL-6 seem to be only partially involved in the pathophysiology of CRPS1, as indicated by the lack of coherence between levels of TNF-α and IL-6 and the signs and symptoms of inflammation and disease duration.

Other inflammatory mediators, a combination of mediators, or even other mechanisms may, however, play a greater role in the pathophysiology of CRPS1 and explain in part the course of the disease. Amino acids, for example, could also be responsible for the characteristics of inflammation, as discussed in the Introduction. In Chapter 4, we investigated the role of amino acids in the pathogenesis of CRPS1. This study shows for the first time a pronounced increase in plasma amino acid levels in 64 CRPS1 patients of the initial 66. The marked differences in glutamate, glutamine, glycine, taurine, and arginine levels between these patients and 51 age- and sex-matched healthy controls suggest the involvement of both the endothelium-dependent arginine-nitric oxide system and the NDMA receptor in CRPS1. However, no proof could be provided that the changes in plasma amino acids are causally related to the pathogenesis of CRPS1. We found no correlations between measures of pain and indicators of psycho neuroticism and emotional instability. In Chapter 5, we report the results of an investigation of the role of serotonin (5-HT) with a study of 35 CRPS1 patients of the original 66 versus 35 age-matched healthy controls. Markedly elevated plasma 5-HT concentrations were found, suggesting a role for 5-HT in patients with CRPS1. However, because of a lack of correlation between 5-HT patterns and distinct disease characteristics, 5-HT is probably one of a number of mediators in the cascade of processes that occur in the course of this painful and debilitating disease.

Chapter 6 describes our investigation into the involvement of mediators in CRPS1 by measurement of several other cytokines using new detection techniques that enable multiple cytokine measurement in small samples. With the use of new techniques, the multiplex-25 bead array cytokine assay and LuminexTM technology, 10 representative cytokines were detectable. In addition to TNF-α and IL-6, IL-1RA, IL-8, IL-12, MCP-1, and MIP-1β were detectable, and all were increased in blister fluid from the CRPS1-affected extremities; IP-10 and eotaxin were detectable and diminished in CRPS1. Multiplex bead array assays are useful systems to establish the involvement of cytokines in inflammatory processes by measurements in blister fluids of CRPS1. However, until now it has been unknown whether the newfound mediators could explain the clinical signs and symptoms of CRPS1.

Earlier studies showed that tryptase, a marker for mast cells, was

increased in CRPS1 patients. Mast cells seem to be involved in inflammatory reactions during CRPS1 development and could play a role in the production of cytokines in CRPS1. Mast cells are also involved in allergic reactions. Chapter 7 reports on our investigations of the hypothesis that allergic disease/hypersensitivity could play a role in the initiation or predisposition of CRPS1. However, based on medical history, patient responses to an allergy questionnaire, and objective laboratory findings, we concluded that hypersensitivity was not more common in a group of 66 CRPS1 patients than in the general population. Our findings indicated that IgE-mediated hypersensitivity is not an explanation for the initiation of or predisposition to CRPS1.

From all the findings described in this thesis and summarized in Table 1, we conclude that TNF-α and IL-6 and glutamate, glutamine, arginine, taurine, glycine, and serotonin are partially involved in CRPS1 but that they are individually not responsible for the signs and symptoms of the disease. Other mediators, cells and/or mechanisms, known and unknown, likely combine with those described here to initiate, continue and/or elicit changes in the clinical signs and symptoms of CRPS1 during the course of the disease (General discussion).

Table 1. Summary of the findings in this thesis

- In all stages of the disease, about half of the CRPS1 patients show signs and symptoms of inflammation.
- The proinflammatory cytokines TNF-α and IL-6 are increased in blister fluid of the CRPS1 extremity in the first 2 years of the disease. These mediators are not specifically responsible for the clinical features of CRPS1, which exhibit characteristics of inflammation.
- Levels of TNF-α and IL-6 in blister fluid are diminished 6 years after the initial event. The clinical features pain, temperature, and volume are not improved, but mobility is. Cytokines and the disease characteristics are not correlated.
- The endothelium-dependent amino acids arginine and the NMDA-receptor–related amino acids glutamate, glutamine, and glycine are changed in plasma of CRPS1 patients versus healthy controls. In addition, arginine, taurine, and serotonin are increased in plasma of these patients. The amino acid levels are not correlated with clinical signs and symptoms of CRPS1.
- Patients with CRPS1 are not more hypersensitive to aeroallergens and food allergens than the general Dutch population.

Samenvatting

Samenvatting
Complex regionaal pijn syndroom (CRPS1) is een aandoening van een extremiteit die gewoonlijk ontstaat na chirurgie of een trauma. Echter spontaan ontstaan is ook beschreven. De belangrijke symptomen die in de initiële fase van CRPS1 te zien zijn lijken op de karakteristieken van ontsteking, zoals pijn, verhoogde temperatuur en kleur veranderingen van de huid, zwelling van de extremiteit en functieverlies. Onze hypothese was dat een variatie van ontstekingsmediatoren aanwezig moet zijn in weefselvloeistof van CRPS1 patiënten. Deze mediatoren zouden verantwoordelijk kunnen zijn voor het ontstekingsproces en als gevolg daarvan de symptomen kunnen veroorzaken die vaak bij CRPS1 worden gezien. Bovendien werd verondersteld dat het ontstekingsproces vermindert in de loop van de tijd door normalisatie van de ontstekingsmediatoren. Dit was gebaseerd op veranderingen van klinische symtomen die gezien werden bij CRPS1 patienten gedurende het beloop van de ziekte.
Uit een eerdere studie is gebleken dat de pro-inflammatoire cytokinen TNF-α en IL-6 niet veranderd zijn in plasma van CRPS1 patiënten. Vanwege deze bevinding en het feit dat de symptomen van CRPS1 lokaal in een extremiteit gezien worden, zijn deze twee mediatoren gemeten in lokale weefselvloeistof. Hoofdstuk 2 beschrijft metingen van de levels van TNF-α en IL-6 in kunstmatig gevormd blaarvocht. De levels gemeten in de eerste 2 jaar van de ziekte waren significant verhoogd in de CRPS1 extremiteit in vergelijking tot de contralaterale zijde van 66 CRPS1 patiënten. Slechts ongeveer de helft van de patiënten liet kenmerken van ontsteking zien. Er werd geen correlatie gevonden tussen de cytokinen en de karakteristieken pijn, temperatuur, volume, mobiliteit of de duur van de ziekte.
Hoofdstuk 3 beschrijft de bevindingen die gedaan zijn 6 jaar na het ontstaan van de ziekte. Deze laten zien dat de levels van TNF-α en IL-6 verminderd zijn in de CRPS1 extremiteit van 12 patiënten van de oorspronkelijke 66. Echter pijn, temperatuur en volume zijn niet veranderd. De mobiliteit is wel verbeterd na 6 jaar. Kenmerken van ontsteking waren nog steeds aanwezig in deze fase van de ziekte. Opnieuw werd er geen correlatie gevonden tussen de levels van de cytokinen en de symptomen van CRPS1 gedurende het beloop van ziekte. Uit de bevindingen van deze twee studies kunnen we concluderen dat de pro-inflammatoire cytokinen TNF-α en IL-6 alleen deels betrokken lijken te zijn bij

de pathofysiologie. Dit vanwege het ontbreken van een correlatie tussen de levels van de cytokinen en de symptomen van inflammatie en de duur van de ziekte. Mogelijk spelen andere ontstekingsmediatoren, een combinatie van mediatoren of zelfs andere mechanismen een grotere rol in de pathofysiologie van CRPS1 en kunnen deze deels het beloop van de ziekte verklaren.
Zoals besproken is in de introductie, kunnen aminozuren ook verantwoordelijk zijn voor de kenmerken van ontsteking. In Hoofdstuk 4 hebben we de rol van aminozuren in de pathogenese van CRPS1 onderzocht. Deze studie laat voor het eerst een aanzienlijke verhoging van aminozuur levels zien in 64 CRPS1 patiënten van de oorspronkelijke 66. De duidelijke verschillen in levels van glutamaat, glutamine, glycine, taurine en arginine tussen deze patiënten en 51 gezonde controles, gematched op leeftijd en geslacht, suggereren de betrokkenheid van zowel het endotheel-afhankelijke arginine-stikstofoxide systeem als ook de NMDA-receptor in CRPS1. Echter, er is geen bewijs gevonden dat de veranderingen van aminozuren in plasma oorzakelijk gerelateerd zijn aan de pathogenese van CRPS1. Er is namelijk geen correlatie gevonden met pijn en indicatoren van psychoneurose en emotionele instabiliteit. In hoofdstuk 5 zijn de resultaten beschreven van een onderzoek naar de rol van serotonine in 35 CRPS1 patiënten tegenover 35 gezonde controles, gematched op leeftijd. Er werden duidelijk verhoogde plasma concentraties gevonden. Deze bevinding suggereert een rol voor 5-HT in patiënten met CRPS1. Echter, vanwege het ontbreken van een correlatie met de opvallende karakteristieken van de ziekte, is 5-HT waarschijnlijk een van de vele mediatoren in de cascade van processen die voorkomen gedurende de pijnlijke en invaliderende ziekte.
Hoofdstuk 6 beschrijft het onderzoek naar de betrokkenheid van mediatoren in CRPS1 door verschillende andere cytokinen te meten met nieuwe detectie methoden. Deze methoden maken het mogelijk om meerdere cytokinen te meten in een kleine hoeveelheid blaarvocht. Met het gebruik van deze nieuwe technieken, the multiplex-25 bead array en de LuminexTM technologie, was het mogelijk om 10 representatieve cytokinen te detecteren. Behalve TNF-α en IL-6 waren ook IL-1RA, IL-8, IL-12, MCP-1 en MIP-1β detecteerbaar. Zij waren allemaal verhoogd in blaarvocht van de CRPS1 extremiteit, terwijl IP-10 en eotoxine verlaagd waren in CRPS1. Multiplex bead arrays zijn bruikbare systemen

om de betrokkenheid van cytokinen in ontstekingsprocessen vast te stellen door deze te meten in blaarvocht van CRPS1 patiënten. Het is echter tot nu toe niet bekend of de nieuwe mediatoren de klinische symptomen van CRPS1 kunnen verklaren.
Eerdere studies lieten zien dat tryptase, een marker voor mestcellen, verhoogd was in CRPS1 patiënten. Mestcellen lijken betrokken te zijn bij ontstekingsreacties in CRPS1 en zouden een rol kunnen spelen bij de productie van cytokinen bij deze aandoening. Mestcellen zijn tevens betrokken bij allergische reacties. Hoofdstuk 7 wordt het onderzoek beschreven naar de hypothese dat allergische aandoeningen/hypersensitiviteit een rol kunnen spelen bij het ontstaan van of predispositie voor CRPS1. Echter, gebaseerd op de medische voorgeschiedenis, antwoorden op een allergie vragenlijst en objectiveerbare laboratorium bevindingen kunnen we concluderen dat hypersensitiviteit niet vaker voorkomt bij de groep van 66 CRPS1 patiënten dan bij de algemene Nederlandse bevolking. De bevindingen indiceren dat Ig-E gemedieerde hypersensitiviteit geen verklaring is voor het ontstaan van of de predispositie voor CRPS1.
Uit alle bevindingen beschreven in deze thesis en samengevat in Tabel 1, concluderen we dat TNF-α en IL-6, glutamaat, glutamine, arginine, taurine, glycine en serotonine deels betrokken zijn bij CRPS1, maar deze mediatoren zijn individueel niet verantwoordelijk voor de symptomen van de ziekte. De bekende, of mogelijk nog onbekende mediatoren, cellen en/of mechanismen of een combinatie hiervan kunnen een verklaring zijn voor het ontstaan, het continueren en/of het veranderen van de klinische symptomen van CRPS1 gedurende het beloop van de ziekte (General discussion).

Tabel 1. Samenvatting van de bevindingen in deze thesis.

- In alle fasen van de ziekte laat ongeveer de helft van de CRPS1 patienten kenmerken van ontsteking zien.
- De pro-inflammatoire cytokinen TNF-α and IL-6 zijn verhoogd in blaarvocht van de CRPS1 extremiteit in de eerste 2 jaar van de ziekte. Deze mediatoren zijn niet specifiek verantwoordelijk voor de klinische symptomen van CRPS1, die lijken op de karakteristieken van ontsteking.
- De levels van TNF-α and IL-6 in blaarvocht zijn verminderd 6 jaar na het ontstaan van de ziekte. De klinische symptomen pijn, temperatuur en volume zijn niet verbeterd, mobiliteit is wel verbeterd. De cytokinen en de karakteristieken zijn niet gecorreleerd.
- Het endotheel afhankelijke aminozuur arginine en aan de NMDA-receptor gerelateerde aminozuren glutamaat, glutamine en glycine zijn veranderd in plasma van CRPS1 patienten in vergelijking met gezonde controles. Tevens zij arginine, taurine en serotonine verhoogd in plasma van deze patienten. De aminozuren zijn niet gecorreleerd met de klinische symptomen van CRPS1.
- Patienten met CRPS1 zijn niet overgevoeliger voor inhalatie en voedsel allergenen dan de algemene Nederlandse bevolking.

List of publications

List of publications

Heijmans-Antonissen, C., F. Wesseldijk, R.J. Munnikes, F.J. Huygen, P. van der Meijden, W.C. Hop, H. Hooijkaas, and F.J. Zijlstra, *Multiplex bead array assay for detection of 25 soluble cytokines in blister fluid of patients with complex regional pain syndrome type 1.* Mediators Inflamm, 2006(1): p. 28398.

Kool, J., L. Reubsaet, F. Wesseldijk, R.T. Maravilha, M.W. Pinkse, C.S. D'Santos, J.J. van Hilten, F.J. Zijlstra, and A.J. Heck, *Suction blister fluid as potential body fluid for biomarker proteins.* Proteomics, 2007. 7(20): p. 3638-50.

Groeneweg, G., S. Niehof, F. Wesseldijk, F.J. Huygen, and F.J. Zijlstra, *Vasodilative effect of isosorbide dinitrate ointment in complex regional pain syndrome type 1.* Clin J Pain, 2008. 24(1): p. 89-92.

Wesseldijk, F., F.J. Huygen, C. Heijmans-Antonissen, S.P. Niehof, and F.J. Zijlstra, *Tumor necrosis factor-alpha and interleukin-6 are not correlated with the characteristics of Complex Regional Pain Syndrome type 1 in 66 patients.* Eur J Pain, 2008. 12(6): p. 716-21.

Wesseldijk, F., F.J. Huygen, C. Heijmans-Antonissen, S.P. Niehof, and F.J. Zijlstra, *Six years follow-up of the levels of TNF-alpha and IL-6 in patients with complex regional pain syndrome type 1.* Mediators Inflamm, 2008. 2008: p. 469439.

Wesseldijk, F., D. Fekkes, F.J. Huygen, M. van de Heide-Mulder, and F.J. Zijlstra, *Increased plasma glutamate, glycine, and arginine levels in complex regional pain syndrome type 1.* Acta Anaesthesiol Scand, 2008. 52(5): p. 688-94.

Wesseldijk, F., D. Fekkes, F.J. Huygen, E. Bogaerts-Taal, and F.J. Zijlstra, Increased plasma serotonin in complex regional pain syndrome type 1. Anesth Analg, 2008. 106(6): p. 1862-7.

Wesseldijk, F., A. van Toorenenbergen, R. Gerth van Wijk, F.J. Huygen, and F.J. Zijlstra, *IgE-mediated hypersensitivity: Patients with Complex Regional Pain Syndrome type 1 versus the Dutch population.* A retrospective study Pain Med, June 2008. Accepted for publication.

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Dikke x

Feik

Curriculum Vitae

Curriculum Vitae

Feikje Wesseldijk werd geboren op 17 juli 1979 te Nijmegen. Het VWO examen atheneum werd behaald in 1998 aan het Canisius College Mater Dei te Nijmegen. Na uitgeloot te zijn voor geneeskunde, heeft zij in 1999 haar propedeuse Bouwkunde behaald aan de Technische Universiteit te Delft, om vervolgens dat jaar alsnog te starten met de opleiding geneeskunde aan de Erasmus Universiteit te Rotterdam. Zij haalde haar doctoraal examen in 2003 na een wetenschappelijke stage naar acupunctuur als behandeling voor CRPS1 op het Pijnbehandelcentrum, afdeling Anesthesiologie van het Erasmus MC. Na twee jaar klinische stage, werd in oktober 2005 het artsexamen behaald. Tijdens deze klinische stage werd haar gevraagd of ze opnieuw onderzoek zou willen komen doen op het Pijnbehandelcentrum, afdeling Anesthesiologie van het Erasmus MC. Daar is zij in oktober 2005 gestart met haar promotie onderzoek, gefinancierd door het Ministerie van Economische Zaken, binnen een consortium (TREND) voor onderzoek naar CRPS. Zij is in juli 2008 begonnen met haar opleiding tot anesthesioloog in het Erasmus MC.

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