Effect of Cyclosporin and Amlodipine on Growth and Collagen Production of Human Gingival Fibroblasts

by

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Summary

Drug-induced gingival overgrowth is a disfiguring condition that is a side effect encountered in susceptible responder patients common to three groups of drugs – immunosupressants, calcium channel blockers and anticonvulsant agents. The altered overgrown gingiva can be aesthetically displeasing but in severe cases it can cause functional problems and such patients may eventually require excision of excess tissue. The underlying mechanisms that mediate drug-induced gingival overgrowth is uncertain and the various investigations into the pathogenesis of this disease suggest that it is multifactorial. This study investigated the effects of exogenous addition of cyclosporin and amlodipine on the growth and proliferation of human gingival fibroblasts and the production of collagen by these cells. Results showed that these drugs have a direct stimulatory effect on the gingival fibroblasts of responder patients *in vitro* and there seems to be a synergistic effect between the two drugs. Findings of this study have important relevance as it suggests that fibroblast proliferation and collagen production must play a significant role in the pathogenesis of drug-induced gingival overgrowth.

'Samevatting'

Geneesmiddel geïnduseerde oorgroei van gingivale weefsel is 'n afwyking wat waargeneem word by 'n spesifieke groep 'responder' pasiënte as 'n newe-effek van die gebruik van spesifieke geneesmiddels. Drie groepe geneesmiddels kan hierdie newe-effek tot gevolg hê, nl. Immuunonderdrukkers, kalsiumblokkeerders en antikonvulsie middels. Die veranderde groeipatroon van die gingivale weefsel is esteties onaanvaarbaar en kan ook in ernstige gevalle funksionele probleme veroorsaak wat dit noodsaaklik maak dat hierdie weefsel sjirurgies verwyder moet word. Die meganisme waardeur hierdie oorgroei ontstaan is onseker en navorsing dui daarop dat dit multifaktoriaal van aard is. Hierdie studie het die effek van die eksogene toevoeging van siklosporien en amlodipien op groei en vermeerdering van fibroblaste, sowel as kollageen produksie deur hierdie organismes bepaal. Resultate het getoon dat beide middels *in vitro* 'n direkte stimmulerende effek op die gingivale fibroblaste vanaf 'responder' pasiënte het en dat die twee middels saam 'n sinergistiese effek het. Bevindinge van hierdie studie is belangrik vir die patogenese van geneesmiddel geïnduseerde gingivale oorgroei, want vermeerdering van fibroblaste en produksie van kollageen word deur die middels gestimuleer.

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CHAPTER 1: INTRODUCTION

Gingival overgrowth is a disfiguring condition that may be caused as a result of various interactions between the tissue of the periodontium and external and internal factors. It may be genetic in origin, may appear as part of a more widespread disorder caused by abnormal deposits of various materials or may be acquired – often as a consequence of exposure to drugs (Gokbuget *et al.*, 1997).

Three different groups of drugs have been associated with the occurrence of gingival overgrowth in susceptible individuals. These drugs include immunosuppressants, calcium channel blockers and anticonvulsant agents. Immunosupressants are drugs used in organ transplant patients to prevent graft rejection and in the treatment of a wide variety of other systemic diseases with immunologic components. Calcium channel blockers are extensively used in the field of cardio-vascular therapeutics and anticonvulsant drugs are widely used for the control of seizures in epileptic patients.

A summary of drugs known to induce gingival overgrowth is given in Table 1.1.

For drug induced gingival overgrowth, patients are clinically divided into two categories. In **responders**, an enlargement of the gingiva develops, and as the enlargement progresses, it takes on a lobulated appearance (Chee & Jansen, 1994). The overgrowth predominantly affects the interdental papillae most

Table 1.1 The three groups of drugs known to cause gingival enlargement.

DRUGS	FIRST AUTHOR	MEAN % PREVALENCE OF OVERGROWTH
• **Immunosuppressants**		
- Cyclosporin	Rateitschak-Pluss *et al.*, 1983	25%
- Tacrolimus	Mihatsch *et al.*, 1998	
• **Calcium channel blockers**		
- Nifedipine	Ramon *et al.*, 1984	42%
- Diltiazem	Colvard *et al.*, 1986	
- Nitrendipine	Brown *et al.*, 1990	
- Felodipine	Lombardi *et al.*, 1991	
- Verapamil	Pernu *et al.*, 1989	
- Amlodipine	Barclay *et al.*, 1992	
- Oxodipine	Waner *et al.*, 1988	
• **Anticonvulsant agents**		
- Phenytoin	Kimball, 1939	50%
- Valproic acid	Anderson *et al.*, 1997	
- Phenobarbital	Gregoriou *et al.*, 1996	
- Vigabatrim	Katz *et al.*, 1997	

frequently on the labial and buccal gingiva of both jaws, but also palatal and distal areas surrounding the maxillary molars and premolars. In **non-responders** such lesions are absent.

The altered gingival anatomy caused by overgrowth can be aesthetically displeasing and the formation of tissue pockets can cause discomfort and functional problems during mastication. The overgrowth interfere with proper oral hygiene, contribute to dental carries, and in severe cases even compromises the patient's ability to speak properly (Jackson & Babich, 1997). Those who develop severe overgrowth may eventually require excision of excess tissue during invasive oral surgery. The non-surgical management of the overgrowth by implementing zealous oral hygiene measures has been emphasised (Pihlstrom *et al.,* 1980; Rateitschak-Plüss *et al.,* 1983; Modeér & Dahllöf, 1987; Hancock & Swan, 1992; Kilpatrick *et al.,* 1997; Meraw & Sheridan, 1998) and medicinal treatment by the use of antibiotics (Gomez *et al.,* 1997; Nash & Zaltzman, 1998; Santi & Bral, 1998; Palomar et al., 1998) or mouth rinses (Pilatti & Sampaio, 1997; Santi & Bral, 1998) have been proposed.

Despite a large number of intense clinical and laboratory investigations, the underlying pathogenic mechanisms that mediate drug-induced gingival overgrowth in affected individuals is uncertain and there appears to be no unifying hypothesis on the determinants for the expression of this unwanted effect.

Most histological studies indicate that the appearance of gingival overgrowth has common characteristics for all drug-induced enlargements such as an increase in extracellular ground substance and/or number of fibroblasts (Hallmon & Rossmann, 1999). Consequently resident gingival fibroblasts, which regulate extracellular matrix turnover, are thought to play a central role in the condition. Hassell & Hefti (1991) proposed that normal human gingiva contains several or many phenotypically distinct different subpopulations of fibroblasts and that the clinical appearance and histologic features of the gingiva are a reflection of such populations. A variable in the pathogenesis of drug-induced overgrowth to consider may therefore be the existence of functionally heterogeneous subpopulations of connective tissue cells with genetically-predetermined drug-susceptible gingival fibroblasts. It has been speculated that there is probably a trough or minimal threshold dose of drugs below which gingival overgrowth does not occur. Some baseline drug concentration within the gingival tissues is required to 'initiate' the gingival changes. This threshold dose differs for different subpopulations of fibroblasts and for different individuals (Daley *et al.,* 1986; McGaw & Porter, 1988).

Studies on collagen production by fibroblasts present in overgrown gingival tissue show controversial results. Some investigators found increased collagen production whereas increased non-collagenous matrix deposition within overgrown gingival tissue has also been reported. The increase in connective tissue volume in drug-induced gingival overgrowth has also been explained by a decrease in collagen degradation due to decreased rate of collagenase activity

(See 2.5.1, p22 and 2.5.2, p23). Reports on the different types of collagen produced in overgrown gingival tissue are limited and contradictory.

Studies indicate that children and adolescents appear more susceptible to drug-induced gingival overgrowth than adults. This could be due to a decrease in the metabolic processes involved in gingival overgrowth with age (Morisaki *et al.*, 1993; Nishikawa *et al.*, 1996) or an indication that fibroblasts of the immature individual are more sensitive to the effects of overgrowth-inducing drugs (Allman *et al.*, 1994). Several investigators found males to have a higher occurrence of drug associated gingival overgrowth (Hassell *et al.*, 1984; Ellis *et al.*, 1999) and it has been proposed that plasma testosterone levels could be altered by these drugs (Dayan *et al.*, 1998) (see 2.5.6, p30).

In recent years, cellular and molecular biological techniques have elucidated a variety of growth factors that control connective tissue homeostasis. The major members of these groups include platelet-derived growth factor (PDGF-β), basic fibroblast growth factor (bFGF), interleukin-1 beta (IL-1-β) and interleukin-6 (IL-6). The activation of these growth factors is thought to play an important role in the pathogenesis of drug-induced overgrowth by altering the gingival connective tissue homeostasis (Iacopino *et al.*, 1997; Sasaki & Maita, 1998; Myrillas *et al.*, 1999) (see 2.5.8, p32).

The effect of multiple therapy on the expression of drug-induced gingival overgrowth has received considerable attention as patients taking cyclosporin

often develop high blood pressure that is subsequently treated with calcium channel blockers. The results of most studies showed more *severe* gingival changes and the *incidence* of clinically significant gingival overgrowth was found to be higher in patients receiving concomitant treatment with cyclosporin and nifedipine. Multiple anticonvulsant therapy has also been indicated to lead to an increase in the incidence of gingival overgrowth in paediatric epileptic patients (Maguire *et al.*, 1986) but not in adult epileptics (Kamali *et al.*, 1999) (see 2.5.9, p34).

This study was undertaken to investigate the effects of exogenous addition of two drugs (cyclosporin and amlodipine), that are known to cause overgrowth, on human gingival fibroblasts. Growth and proliferation rates and the production of types IV, V and VI collagens were determined for gingival fibroblasts from responder and non-responder transplant patients. Results obtained were processed, growth curves compiled and the surface areas covered by different types of collagens were determined.

Results showed that an exogenous addition of these two drugs have a direct effect on the gingival fibroblasts of responder patients by stimulating the proliferation of gingival fibroblasts. The effect of combining the treatment cyclosporin with amlodipine was stronger than that of cyclosporin alone, confirming a synergistic relationship. Significant higher coverage by collagen type VI for all three the patient and treatment groups was found. Collagen type VI production by the human gingival fibroblasts derived from the responder

patients was found to be higher when these cells were exposed to cyclosporin and a combination of cyclosporin and amlodipine. Collagen type VI production by the human gingival fibroblasts derived from the non-responder patients was significantly higher when these cells were exposed to cyclosporin only (see Chapter 5, p55).

The results of this study suggest that the fibroblasts must play a significant role in the pathogenesis of gingival overgrowth and that a combination of cyclosporin and amlodipine alter collagen production and therefore attribute to gingival overgrowth.

CHAPTER 2: LITERATURE REVIEW

The essential feature of all drug-induced gingival overgrowth is an increase in the connective tissue matrix, thus drug-induced changes in connective tissue homeastasis is a topic that has been extensively researched. As gingival fibroblasts regulate extracellular matrix turnover, attention is given to these cells and the production of collagen in the first part of this review, whilst the effect of drugs, factors influencing overgrowth and the treatment of overgrowth are also discussed.

2.1 THE FIBROBLASTS

2.1.1 Structure and physiology of fibroblasts

Fibroblasts (Figure 2.1) are the predominant cells of connective tissue and have the ability to synthesise and secrete a wide range of extracellular molecules including collagens - which are of particular interest in this study - as well as elastins, proteoglycans, and glycoproteins (McCulloch & Borden, 1991). Under the light microscope, fibroblasts are normally recognised by their association with collagen fibres. The resting fibroblast has a flattened, dark-staining, closed nucleus and little cytoplasm. The active fibroblast has a pale staining open-faced nucleus and much more cytoplasm. Under the electron microscope, active fibroblasts are seen to have the usual complement of cytoplasmic organelles, but in exaggerated amounts, so that there are a number of the Golgi complexes and many profiles of rough endoplasmic reticulum, mitochondria,

and secretory vesicles, all indicative of these cells' synthetic and secretory function (Ten Cate, 1994).

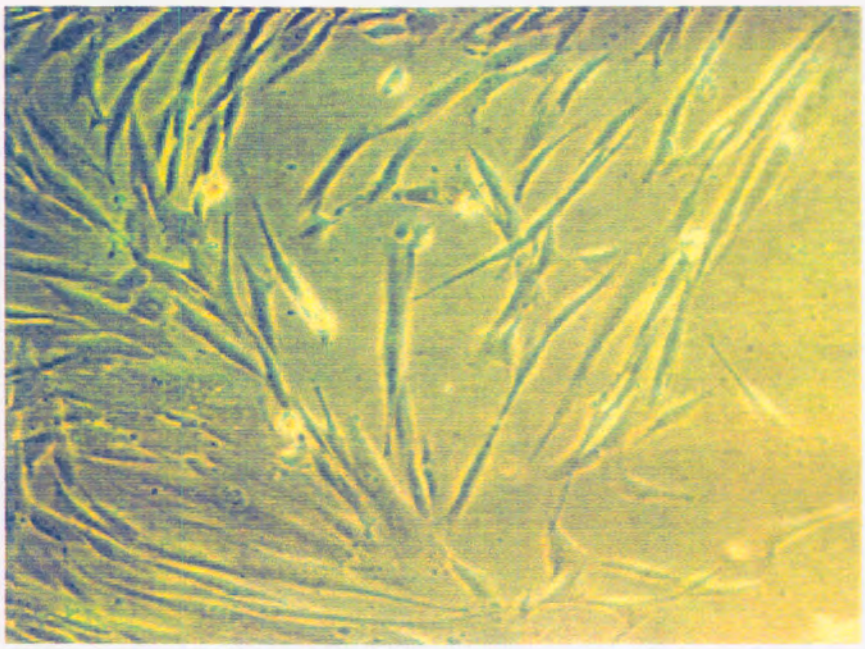

Figure 2.1 The appearance of human gingival fibroblasts as seen under the light microscope (100 X)

In human gingiva, fibroblasts deposit and maintain the dense fibrous connective tissue and evidence exists that these cells regulate their own proliferation autocrinically (Hanazwa *et al.,* 1988). Collagen that is synthesised by gingival fibroblasts exhibits an unique pattern of organisation and serves functionally as a very important structural component which maintain the organic union between the soft tissue and the calcified tooth surface (Narayan & Page, 1976; Narayan *et al.,* 1988).

2.1.2 Fibroblasts at the wound site

Metabolism of fibroblasts plays a key role in wound healing. Once the inflammatory process has been initiated, the macrophage responds to and processes intracellular signals, amplifies the signals and transmits the signals to fibroblasts, endothelial cells, and vascular smooth muscle cells (Clark & Henson, 1988). It is believed that the macrophages release specialised cytokines termed polypeptide growth factors. The major members of these groups include platelet-derived growth factor (PDGF), transforming growth factor beta (TGF-β), and basic fibroblast growth factor (bFGF). These act as mitogens stimulating migration/proliferation of fibroblasts, angiogenesis factors facilitating neovascularization, and factors which control the synthesis of connective tissue proteins (Martin *et al.,* 1992; Kiristy *et al.,* 1993). Other mentioned growth factors involved in connective tissue turnover are connective tissue growth factor (CTGF) (Uzel *et al.,* 2001) and keratinocyte growth factor (KGF) (Das & Olsen, 2000).

The presence of myofibroblasts is a well-established feature of wound healing (Lipper *et al.,* 1980). Myofibroblasts are associated with the later stages of tissue turnover, specifically with the transition from the granulation to the remodelling phases (tissue contraction) of the wound healing process (Clark & Henson, 1988).

2.2 COLLAGEN

2.2.1 Types of collagen

Collagen is the principal extracellular constituent of most connective tissues and is responsible for the structural and functional integrity. Human gingiva is an organised connective tissue made up of several distinct types of collagen fibres and quantitative differences in the proportions of different collagen types in local anatomic sites exist (Bornstein & Sage, 1980). Table 2.1 shows the known types of collagen types as well as the most abundant locations of these collagens.

2.2.2 Collagen structure and formation

All collagen types are composed of three polypeptide alpha chains (α chains) coiled around each other to form a typical triple helix configuration (Leblond, 1989). Collagen, as other proteins, begins to be formed within the fibroblasts when specific amino acids are linked together into individual polypeptide chains on ribosomes associated with the rough endoplasmic reticulum (RER). The peptide chains are segregated out of the ground cytoplasm into the RER and then moved through its cisternae to the region of the Golgi complex where their orderly packaging takes place. Before helix formation can occur, there is a requirement for hydroxylation of some of the proline and lysine residues within the polypeptide chains to form hydroxyproline and hydroxylysine. Once

Table 2.1 Summary of the known collagen types and their locations (Ten Cate , 1994).

TYPE	LOCATION
I	Abundant in skin, bone, gingiva, periodontal ligament, cementum and most other connective tissue.
II	Cartilage, vitreous humor.
III	Embryonic connective tissue, pulp, periodontal ligament, skin.
IV	Basement membrane.
V	Basement membranes, blood vessels, ligaments, skin, dentin, gingiva, periodontal ligament.
VI	Ligaments, gingiva, skin, bone cartilage.
VII	Anchoring fibrils of basement membrane.
VIII	Endothelial cells, cartilage, junctional epithelium
IX	Cartilage
X	Cartilage, bone
XI	Cartilage, bone
XII	Calvaria, tendon, periodontal ligament, cartilage.
XIII	Epidermis, cartilage

hydroxylated and glycosylated, the three polypeptide chains are assembled into the classic triple helix configuration. Once aligned and assembled into the helix configuration, the molecule is transported to the Golgi apparatus, where further remodelling occurs to form a procollagen molecule. The procollagen molecules are collected in Golgi vacuoles and subsequently exocytosed or secreted out of the cell. The procollagen extension peptides are removed, and when sufficient numbers have been attained, collagen molecules are assembled extracellularly

so that they can be observed in the form of the typical banded collagen fibril. Collagen molecules are thus found in the extracellular matrix adjacent to the cell (Weinstock & Leblond, 1974).

2.2.3 Collagen degradation

Physiologically, connective tissue turn-over and remodelling involves a balance between synthetic and degradative activity with fibroblasts having a dual role in the process. They synthesise all the components of connective tissue matrix; but also contribute to degradation via both intracellular and extracellular pathways.

Two mechanisms are associated with the degradation of collagen:

i) The selective ingestion of collagen fibrils by fibroblasts and their degradation intracellularly.

ii) The secretion of a number of enzymes that sequentially degrade collagen extracellularly (Ten Cate 1994).

Matrix metalloendoproteinase is the name given to a group of enzymes secreted by some cells - including fibroblasts - that can degrade collagen and other matrix macromolecules into small peptides. Collagenase is the best characterised of these enzymes. It plays a vital role in collagen degradation, for it is the only enzyme that at a neutral pH can cleave the triple helix of the

collagen molecule. In addition to collagenase, various lysosomal proteinases are thought to contribute to protein turnover and/or be involved in the collagen metabolism (Sodek, 1977). Among the proteinases, medullasin is of special importance. This enzyme is mainly found in granulocytes and is readily secreted into the extracellular space at sites of inflammation. It degrades gingival connective-tissue components such as collagens and proteoglycan. (Ozaki *et al.*, 1998).

2.2.4. Detection of collagen tissue using immunohistological techniques

Immunohistological techniques involve the use of labelled antibodies as specific reagents for the localisation of the tissue constituents (antigens) in situ. Development of antibodies specific for the distinct types of collagen has made it possible to show the distribution of collagenous proteins in human tissue. Antibody molecules consist of four polypeptide chains. There are two identical short strands, called light chains and two identical long strands, called heavy chains. The four chains are held together by disulphide bonds, forming a Y-shaped molecule. An antibody molecule recognises a specific antigen because it possesses two clefts or depressions into which an antigen can fit (Serotec Product Guide, 1998).

Conjugates of an antibody and fluorochrome can be made which upon incubation with cells will label connective tissue components in situ. Structures can be visualised by illuminating the tissue or cells with UV light and observing

and recording the fluorescent image emitted (Malik & Lillehoj, 1994) (See Figure 2.2).

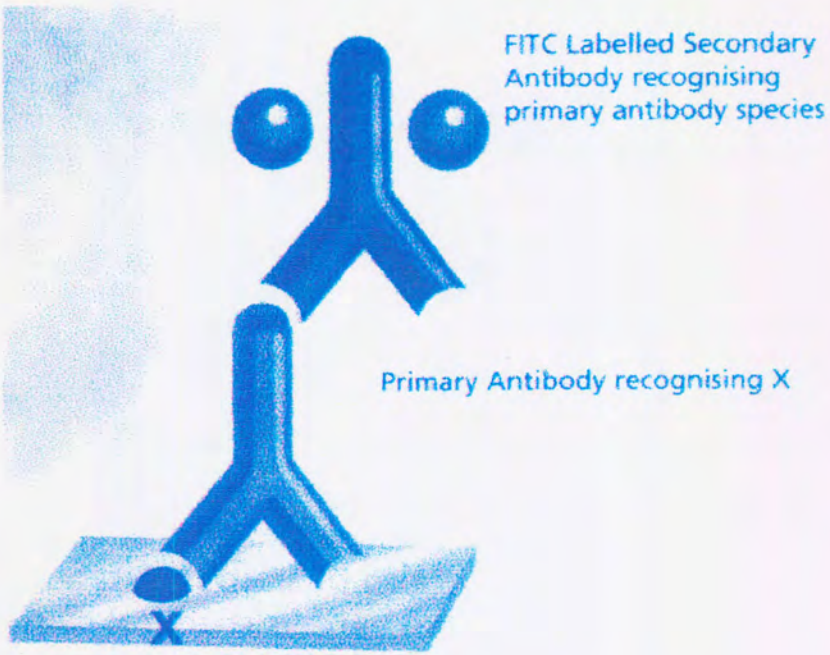

Figure 2.2 Indirect FITC labelling Procedure

2.3 MORPHOLOGY OF GINGIVAL OVERGROWTH

Clinical and histological features of gingival overgrowth induced by all three different groups of drugs have been described as being similar by a number of authors (Rateitchack-Pluss *et al.,* 1983; Wysocki *et al.,* 1983; Butler *et al.,* 1987; Nishikawa *et al.,* 1991; Romanos *et al.,* 1993a; Romanos *et al.,* 1993b; Bullon *et al.,* 1994; Bonnaure-Mallet *et al.,* 1995; Harel-Raviv *et al.,* 1995; Katz *et al.,* 1997).

2.3.1 Clinical features

Drug induced gingival overgrowth usually commences as an interdental papillary enlargement that is usually more pronounced on the labial gingiva of the lower anterior teeth, around the maxillary molars and/or interdental gingiva than on the palatal or lingual surfaces (Ramon *et al.,* 1984; Van der Wall *et al.,* 1985). The papillary enlargement increases, and affected papillae may become enlarged to the point that they touch, resulting in the clinical presence of pseudoclefts. This leads to the gingival tissue appearing lobulated and the surface of the lobulations are pebbly and granular (Khocht & Schneider, 1997). Overgrowth is often restricted to the portion of keratinized gingiva (Friskopp & Klintmalm, 1986) but can also extend coronally to the occlusal surface. Although tissue overgrowth usually diminishes as it approaches the mucogingival junction, coronal progression may partially or totally obscure the crowns (see Figure 2.3) of the teeth (Hallmon & Rossmann, 1999). The enlarged tissues are generally soft, red or bluish-red, extremely fragile and bleed easily upon probing. Surgical removal sometimes demonstrates considerable bleeding due to the increased vascularity of the tissue (Darbar *et al.*, 1996; Gregoriou *et al.,* 1996).

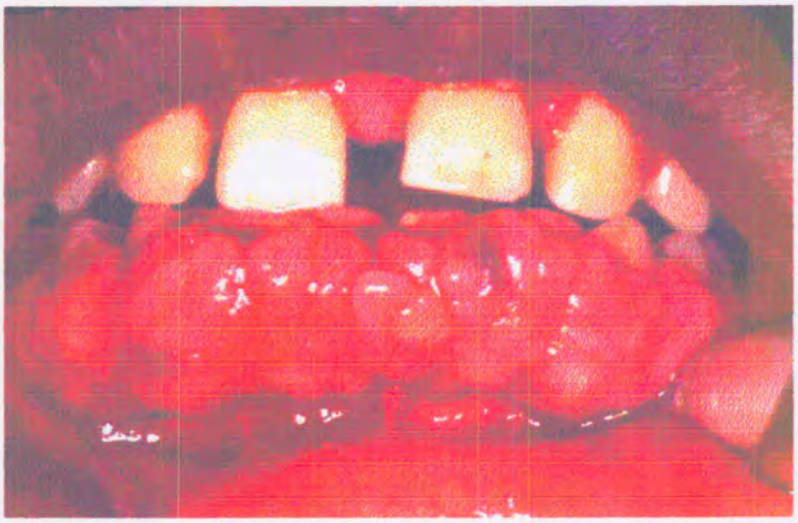

Figure 2.3 A patient on phenytoin treatment. The presence of generalised gingival overgrowth is seen obscuring the clinical crowns.

2.3.2 Histological features

The width of the gingival epithelium is usually several times that of normal epithelium. The gingival epithelium appears parakeratotic and acanthotic with pseudo-epitheliomatous proliferation. Edema and tubular elongation of the rete occur consisting of a few layers of basal cells growing almost vertically into the lamina propria and manifesting deep seated keratinization pegs (Zlotogorski *et al.,* 1989; Gregoriou *et al.,* 1996). Gingival overgrowth has been described as highly vascularized with an increase in the number of capillaries, and focal accumulations of infiltrating inflammatory cells have been seen (Rateitschak-Pluss *et al.,* 1983). Some degree of increased numbers of fibroblasts and/or proliferation of collagen fibres and non-collagenous material have been reported (Lucas *et al.,* 1985; Jones, 1986; Barak *et al.,* 1987). Mariani and co-workers

(1996) found in pathological samples obtained from cyclosporin-treated responder patients that fibroblasts have all the ultrastructural signs that characterise an active state of synthesis and secretion. The ground substance not only fills the tunica propria to saturation, but has pronounced tendency to invade the epithelium and to expand in newly formed intra-epithelial spaces by passing through openings in the basal lamina and by probably breaking up cytoplasmic bridges between adjacent cells. Mast cells are also present in high numbers in overgrowth-affected gingiva, and are particularly abundant along blood vessels.

2.4 GROUPS OF DRUGS ASSOCIATED WITH GINGIVAL OVERGROWTH

2.4.1 Immunosuppressants

Cyclosporin A is produced from a fungus (*Tolypocladium inflatum)* and is a cyclic polypeptide that has reached significant use as a selective immunosuppressant. It is today the most widely prescribed drug to control the rejection of organ transplantation or auto-immune diseases because of its suppressive action on specific T-cell subpopulations and the production of lymphokines (Morisaki *et al.,* 1997). This therapy is however associated with several side effects of which gingival overgrowth is one (Rateitschak-Pluss *et al.,* 1983; Tyldesley & Rotter, 1984; Seymour & Jacobs, 1992; Thomason *et al.,* 1993; Aufricht *et al.,* 1997).

The incidence of cyclosporin-induced gingival overgrowth in humans varies from study to study and has been reported to be approximately 25%, ranging from 8% to 70% in patients treated with the drug (Daley *et al.*, 1986; Seymour *et al.*, 1987; Seymour & Jacobs, 1992; Pernu *et al.*, 1993).

Recently tacrolimus a new immunosuppressive molecule used as a treatment of second choice to control acute corticoresistant rejection, has also been reported to cause gingival overgrowth, however less pronounced than cyclosporin (Mihatsch *et al.*, 1998).

2.4.2 Calcium-Channel Blockers

The calcium-channel blocking group of drugs are extensively used in the treatment of cardiovascular disorders, including angina pectoris, hypertension, coronary artery spasm and cardiac arrhythmia. These drugs act by inhibiting the extracellular calcium influx across the membranes of cardiac and vascular smooth muscles without changing the serum concentration. This reduces the contractile process of the cardiac muscles, thereby reducing the utilisation of oxygen by the myocardium. Concurrently, the vascular smooth muscles of the main coronary and systematic arteries have their contractile process also reduced, resulting in relaxation and prevention of coronary artery spasms (Long, 1984).

Calcium-channel blocking drugs were introduced onto the therapeutics market in 1978; six years later the first case reports describing a disfiguring gingival overgrowth as a side effect of nifedipine therapy appeared in the literature (Lederman *et al.,* 1984; Ramon *et al.,* 1984). Subsequently, numerous reports have linked nifedipine and gingival overgrowth. The reported prevalence of gingival overgrowth varies from 6% to 83% with an average effect approximating 42% (Barak *et al.,* 1987; Zlotogorski *et al.,* 1989; Fattore *et al.,* 1991; Barclay *et al.,* 1992; Nery *et al.,* 1995; Ellis *et al.,* 1999).

A number of cases have also been published describing patients taking amlodipine who demonstrated gingival overgrowth (Barclay *et al.,* 1992; Ellis *et al.,* 1993; Seymour *et al.,* 1994; Salerno *et al.,* 1995, Ellis *et al.,* 1999; Van der Vleuten, 1999). Other calcium channel blockers that have been implicated in gingival overgrowth include diltiazem (Colvard *et al.,* 1986; Bowman *et al.*, 1988; Bullon *et al.*, 1995), verapamil (Pernu *et al.,* 1989), nitrendipine (Brown *et al.,* 1990), felodipine (Lombardi *et al.,* 1991), and oxodipine (Waner *et al.*, 1988; Nyska *et al.,* 1994).

2.4.3. Anticonvulsants

Phenytoin has proved to be one of the most effective drugs available for the control of epileptic seizures since its introduction into medicine in 1938. It selectively depresses the motor cortex of the central nervous system and is believed to mediate this action by stabilising neuronal discharge and limiting the

progression of neuronal excitation by blocking or interfering with calcium influx across cell membranes (Seymour & Heasman, 1988; Leppik, 1990; Rees 1993).

Kimball (1939), was the first to report the peculiar side effect that phenytoin has on the gingival tissue. It has now been shown that the usefulness of phenytoin is limited by a side effect that often leads to a severe overgrowth of gingival tissue in about 40-50% of the patients treated. (Hassell, 1981; Stinnett *et al.*, 1987; Hassell & Hefti, 1991; Casetta *et al.*, 1997).

Valproic acid is an anti-epileptic agent whose exact mechanism is unknown, but its activity may be related to increased brain levels of gamma-amino butyric acid. Anderson and co-workers (1997) reported a patient on valproic acid treatment with gingival overgrowth resembling other drug-induced gingival overgrowths, both in the clinical and histological appearance.

Vigabatrim is a relatively new medication used to treat epilepsy. Katz and co-workers (1997) were the first to report vigabatrim-induced gingival overgrowth of which the histopathologic features are comparable with that of phenytoin-induced gingival overgrowth.

2.5. INTRINSIC AND EXTRINSIC FACTORS INFLUENCING GINGIVAL OVERGROWTH

Factors influencing gingival overgrowth resulting from drug usage show controversial results in studies on all three of the drug groups known to cause overgrowth of the gingiva. Many investigators have investigated the following aspects of drug-induced overgrowth:

2.5.1 Changes in fibroblast proliferation

Increased proliferation rates of fibroblasts isolated from hyperplastic gingival tissue have been shown in reports on all three groups of drugs. (Zebrowski *et al.*,1986; Barak *et al.*,1987; Jacobs *et al.*,1990; Zebrowski *et al.*,1994; Tipton *et al.*, 1997; Sasaki *et al.*, 1998; Uzel *et al.*, 2001). Numerous fibroblasts were seen to be present in cyclosporin-induced enlarged gingival tissue (Mariani *et al.*,1993) and in a study by Breschi and co-workers (2000) the *in vitro* addition of cyclosporin highly stimulated the fibroblasts from cyclosporin-treated patients both in cells from enlarged gingival sites and in cells from clinically healthy gingival sites of the same patients . Fuji and co-workers (1994) demonstrated that cultures of gingival fibroblasts from patients who reacted to nifedipine, cultivated in the presence of calcium-channel blockers or phenytoin, tended to show higher proliferation rates (10,3-17,9%) than those from non-reactive patients. The comparison of responder and non-responder cells by McKevitt and Irwin (1995) showed that at both low and high fetal calf serum levels, cells

from overgrown tissue grew more quickly and to higher saturation cell densities than the non-responder lines.

In contrast, similar research showed that these drugs had no significant affect on gingival fibroblast proliferation rates (Nishikawa *et al.*,1986; Salo *et al.*,1990; Hassell *et al.*, 1991; Barber *et al.*, 1992; Tipton & Dabbous 1993) and a decrease in proliferation rates has also been reported (Nishikawa *et al.*, 1986; Nishikawa *et al.*, 1991). However, Nishikawa and co-workers (1991) used fibroblasts from only one case each of a responder and non-responder, and intact cells from a patient who did not receive nifedipine treatment.

2.5.2 Collagen production

Collagen is the principal non cellular constituent of most connective tissues and is responsible for their structural and functional integrity. Collagen that is synthesised by gingival fibroblasts exhibits an unique pattern of organisation and serves functionally as a very important structural component which maintain the organic union between the soft tissue and the calcified tooth surface (Narayan & Page, 1976; Narayan *et al.*, 1988).

2.5.2.1 Collagen versus ground substance production

Increased total protein and collagen production was shown after incubation of gingival fibroblasts in the presence of high doses of cyclosporin (Wysocki *et al.*,

1983; Zebrowski *et al.,* 1986; Schincaglia *et al.,* 1992), nifedipine (Lucas *et al.,* 1985; Tipton & Dabbous 1993) and phenytoin (Narayanan & Page, 1983; Vernillo & Schwartz, 1987; Nayaranan *et al.,* 1988; Johnston *et al.,* 1990; Hassell & Gilbert, 1993). The collagen synthesis study of Fujii and co-workers (1994) showed cells from nifedipine reactive patients to give 9,6-55,7% greater collagen synthesis rates in respect to control by calcium channel blockers or phenytoin.

On the contrary, increased non-collagenous matrix deposition within overgrown gingival tissue have been reported (Hall & Squier, 1982; Dahllöf *et al.,* 1984; Rostock *et al.,* 1986, Pisanty *et al.,*1988; Salo *et al.,*1990; Bonnaure-Mallet *et al.,* 1995). Mariani and co-workers (1993) observed a scarcity of collagen fibers and a particular abundance of amorphous substance in cyclosporin-induced overgrowth with a marked infiltration of plasma cells. The total increase in gingiva volume could be accounted for by the increase in the amorphous ground substance. These observations have been confirmed by Mariani and co-workers (1996).

2.5.2.2 Types of Collagen

Schneir and co-workers (1978) found that collagen types I, III and V are present in normal ratios in phenytoin-induced overgrowth. Similarly McGaw & Porter (1988) found no difference in the distribution of different types of collagens in cyclosporin-induced gingival overgrowth in comparison with healthy gingiva. In

contrast to these findings Narayanan and Page (1983) found an abnormal ratio of collagen types I:III and demonstrated an increase in type V collagen in enlarged gingiva as a result of the use of phenytoin. In agreement with these findings, Bonnaure-Mallet and co-workers (1995) found an abnormal ratio of types I:III collagen after treatment with the same drug (phenytoin).

The *in vitro* studies of Schincaglia and co-workers (1992) have shown that cyclosporin caused a specific rise in the level of type I procollagen and immunohistological investigations of Bonnaure-Mallet and co-workers (1995) showed a comparatively higher percentage of area occupied by collagen type IV collagen in cyclosporin-induced overgrowth. Immunohistochemical analysis by Kataoka and co-workers (2001) revealed that type I collagen was more prevalent in the connective tissue of nifedipine-treated rat gingiva than controls and they suggested that a decrease in collagen degradation due to lower phagocytosis was closely associated with the increase in type I collagen accumulation.

Romanos and co-workers, (1993a) showed a characteristic pattern of distribution in different extracellular matrix components of the most important gingival overgrowths. They were able to show that extracellular matrix of the gingival alterations has a characteristic topographical distribution in collagens types IV, V, and VI as well as fibronectin. In a similar study, Romanos and co-workers (1993b) demonstrated the localization of collagen types I, III, IV, V, VI

and VII as well as the glycoprotein fibronectin in nifedipine-induced gingival overgrowth.

2.5.2.3 Collagen Degradation

As already mentioned (see 2.2.3) matrix metalloendoproteinase is the name given to a group of enzymes that can degrade collagen and other matrix macromolecules into small peptides.

A number of researchers showed that an increased rate of collagen synthesis coupled with a decreased rate of phagocytosis or reduction in collagenase activity would result in a net increase in the amount of ground substance. This could explain the increase in connective tissue volume in drug induced gingival overgrowth (Hassell, 1982; Moy *et al.,* 1985; Hassell *et al.,* 1988; McGaw & Porter, 1988; Thomason *et al.,* 1993; McKevitt & Irwin, 1995; Tipton *et al.,* 1995).

Ozaki and co-workers (1998) used a rat model of nifedipine-induced gingival overgrowth and investigated the localisation and distribution of medullasin during the development and appearance of the overgrowth. They speculated that *in vitro* medullasin could possibly enhance and stimulate the function of fibroblasts by modulation of cytokines and growth factors following the activation of macrophages or conversely, the enzyme may actively degrade such accumulated collagens.

2.5.3 Glycoaminoglycan content

Zebrowski and co-workers (1994) suggested that increased levels of non-sulfated glycosaminoglycans in tissue can occur with cyclosporin exposure and it has been shown that tissue from phenytoin (Kantor & Hassell, 1983; Dahllöf *et al.*, 1984; Dahllöf *et al.*, 1986) and nifedipine (Lucas *et al.*, 1985; Jones, 1986) induced gingival overgrowths are characterised by increased presence of glycosaminoglycans compared with normal gingival controls.

The biochemical results of Rocha and co-workers (2000) however conflict with the data reported by the above mentioned groups. They found the total and relative amounts of glycosaminoglycans to be similar between normal and overgrown gingiva. These discrepant results suggest that an increase in gingival glycosaminoglycans still remains an open question.

2.5.4 'Cell selection model'

A suggested important variable in the pathogenesis of drug-induced overgrowth to consider may be the existence of functionally heterogeneous subpopulations of connective tissue cells in the gingiva (Hassell *et al.*, 1976; Hassell, 1981; Hassell & Stanek, 1983; Narayanan et al, 1988; Bartold, 1989; De Camargo, 1989; Pagliarini *et al.*, 1995).

Hassell & Hefti (1991) proposed that normal human gingiva contains several or many phenotypically distinct different subpopulations of fibroblasts and that the clinical appearance and histologic features of the gingiva are a reflection of such populations.

Coley and co-workers (1986) demonstrated that the effects of cyclosporin on normal human fibroblast proliferation varied among individual cell strains, where strains were shown to increase, decrease, or remain unchanged.

In a study undertaken by Varga and co-workers (1998), the most important finding was that in a group of transplant patients, all patients exhibiting severe overgrowth after treatment with cyclosporin, had evidence of gingival overgrowth (not associated with poor oral hygiene) *prior* to their transplant. They concluded that a pre-existing hyperplastic response in the gingiva predisposes the patient to the development of severe gingival overgrowth. Genetically-predetermined drug-susceptible subpopulations of gingival fibroblasts have therefore been suggested to play a major role in the control of connective tissue function and the accumulation of connective tissue.

2.5.5 Drug dosage

A positive correlation has been found between plasma cyclosporin levels or total dose of cyclosporin administered and the severity of gingival enlargement (Adams & Davies 1984; Seymour *et al.*, 1987; Seymour & Smith, 1991; Hefti *et*

al., 1994; Somacarrea *et al.*, 1994; Morisaki *et al.*,1997) and dose dependent overgrowth has been shown in patients (Barak *et al.*, 1987) and rats (Fu *et al.*, 1998) receiving nifedipine. A number of workers found a positive relationship between the total level of phenytoin in serum and/or saliva and the incidence and severity of overgrowth (Addy *et al.*, 1983; Perlik *et al.*, 1995).

In contrast it has been reported that no direct correlation exists between the oral dose or blood level of cyclosporin and gingival overgrowth (Daley *et al.*, 1986; Ross *et al.*, 1989; King *et al.*, 1993; Allman *et al.*, 1994; Cebeci *et al.*, 1996; Gomez *et al.*, 1997; Varga *et al.*, 1998). Studies showed nifedipine drug dosage not to be a significant predictor of gingival overgrowth (Barclay *et al.*, 1992; Ellis *et al.*, 1992; Nery *et al.*, 1995) and no or only a weak correlation was found between the degree of gingival overgrowth and phenytoin level (Little *et al.*, 1975; Girgis *et al.*, 1980; Hassell & Hefti, 1991; Thomason *et al.*, 1992; Ball *et al.*, 1996; Sasaki & Maita, 1998).

In agreement with the cell selection model it has further been speculated that a specific threshold exists for susceptibility of the fibroblasts to the effects of these drugs. Some strains of human fibroblasts may respond to lower concentrations of a specific drug, while other strains respond only at higher concentrations. At drug concentrations higher than the threshold concentration, however, all strains of responder patient fibroblasts become susceptible to the effects of the drug (Daley *et al.*, 1986; McGaw & Porter, 1988).

2.5.6 Age, sex and gingival overgrowth

The incidence of gingival overgrowth has been reported to be higher in children than in adults (Stinnett *et al.,* 1987; Morisaki *et al.,* 1993; Somacarrera *et al.*, 1994; Thomason *et al.*, 1995; Aufricht *et al.,* 1997; Nakou *et al.,* 1998). The incidince of cyclosporin-treated children who showed gingival overgrowth after renal transplantion has been reported to be as high as 60% (Aufricht et al., 1997), 70% (Bökenkamp et al., 1994) and 90% (Lowry et al., 1995). It has also been shown that younger age (with children aged less than 10 years at the time of transplantation having the worst overgrowth) seemed to predispose to the severest level of gingival involvement (Casetta *et al.,* 1997; Kilpatrick *et al.,* 1997).

Hassell and Hefti (1991) reported that there was no evidence suggesting that sex or race affect the occurrence of phenytoin associated gingival overgrowth. This contradicts earlier findings by Hassell and co-workers (1984) where they reported males as having a higher prevalence than females.

Dayan and co-workers (1993) found in a preliminary study, that the gingiva of a group of castrated male dogs exposed to oxodipine showed a histological pattern similar to normal gingiva, whereas normal oxodipine-treated male dogs had a different histological pattern compared to normal gingiva. In later studies, they found that castration of beagle dogs correlated with a lack of gingival overgrowth, while testosterone injection to the same dogs was associated with

an increase of the gingival index and gingival overgrowth index. They concluded that androgens, mainly testosterone, play an important role in the pathogenesis of gingival overgrowth (Nyska *et al.*, 1994; Dayan *et.al.,* 1998).

These findings could explain the results of Harel-Raviv and co-workers (1995) and Ellis and co-workers (1999) that males are 3 times more likely to develop overgrowth than females.

Tyldesley and Rotter (1984) however found that female renal transplant patients taking cyclosporin therapy showed a higher incidence of gingival overgrowth than males (38% compared to 17% respectively).

2.5.7 Involvement of dental plaque

Numerous studies have found significant correlations between plaque scores and the occurrence and/or severity of gingival overgrowth (Rateitschak-Plüss *et al.,* 1983; Adams & Davies, 1984; McGaw *et al.,* 1987; Dongari *et al.,* 1993; Rees, 1993; Allman *et al.,* 1994; Bullon *et al.,* 1994; Somacarrera et al., 1994; Fu *et al.,*1997; Nakou *et al.,* 1998; Santi & Bral 1998; Varga *et al.,* 1998, Ellis *et al.,* 1999; Majola *et al.,*2000). In contrast, some reports found plaque control programs to have no or a limited effect on the prevention or lessening of cyclosporin-induced gingival overgrowth (Seymour *et al.,* 1987; Seymour & Smith, 1991) or nifedipine gingival overgrowth (Barclay *et al.,* 1992).

The general consensus is however that zealous oral hygiene measures should be emphasised to prevent or at least manage gingival overgrowth (Pihlstrom *et al.,* 1980; Rateitschak-Plüss *et al.,* 1983; Modeér & Dahllöf, 1987; Hancock & Swan 1992; Kilpatrick *et al.,* 1997; Meraw & Sheridan, 1998).

2.5.8 The role of cytokines in gingival overgrowth

Growth factors have been extensively studied recently, as their activation is thought to play an important role in the pathogenesis of drug-induced overgrowth. It is believed that the macrophages release specialised cytokines termed polypeptide growth factors.

The results of investigations by Iacopino and co-workers (1997) indicated that PDGF-ß mRNA is significantly increased in hyperplastic gingival tissue from phenytoin and cyclosporin-treated patients relative to normal controls independent of the inflammatory state. PDGF-ß is released from fibroblasts amongst others and is a major mitogen and chemoattractant for fibroblasts, stimulating proliferation and synthesis of glycosaminoglycans, fibronectin and collagen. Increased gingival levels of PDGF-ß may therefor promote fibroblast proliferation and production of constituents in gingival overgrowth. Other similar studies endorsed these observations (Dill *et al.,* 1993; Nares *et al.,* 1996; Plemons *et al.,* 1996; Dill & Iacopino, 1997). The net effect of findings of these investigators postulate that the macrophage may play a primary role in drug induced gingival overgrowth through enhanced macrophage PDGF-ß gene

expression rather than an increase in the number of macrophages producing these growth factors.

The underlying mechanism of drug-induced overgrowth has also been explained in terms of increased bFGF levels (Sasaki & Maita, 1998). bFGF stimulates fibroblast proliferation and synthesis of extracellular matrices, such as collagen or proteoglycans. These investigators concluded that daily phenytoin administration was responsible for the enhancement of the serum bFGF level in gingival overgrowth patients and found a significant positive correlation between the degree of gingival overgrowth and the serum bFGF.

A study by Myrillas and co-workers (1999) showed that fibroblasts derived from cyclosporin-induced overgrown gingiva produced significantly higher levels of IL-6 than in inflamed or normal tissue. In contrast IL-1 beta levels in overgrown tissue were not statistically greater than those in inflamed tissue. They concluded that cyclosporin does regulate cytokine expression in gingival tissue and that this effect may play an important role in the pathogenesis of overgrowth. The data from a recent study (Uzel *et al.,* 2001) showed significantly higher CTGF staining in phenytoin-induced gingival overgrowth and it was shown that nifedipine upregulates KGF and gene transcription by gingival fibroblasts *in vitro* (Das & Olsen, 2000).

To date, there are no studies ruling out the possibility of direct interactions between cyclosporin or phenytoin and cytokines. It is possible that the drugs

may bind with these proteins, altering their bioavailablilty and subsequent activity. Thus, it remains to be determined if alterations of cytokine levels by drugs are solely due to effects on gene expression or whether direct protein interactions may also be partially responsible for these cytokine alterations.

2.5.9 The effect of multiple therapy on the expression of drug-induced gingival overgrowth

2.5.9.1 Interaction between cyclosporin and calcium channel blockers

Patients taking cyclosporin often develop high blood pressure that is subsequently treated with nifedipine (Thomason *et al.,* 1995; Margiotla *et al.,* 1996). Combinations of these drugs are routinely prescribed for patients who have undergone a renal transplant, because nifedipine, in addition to controlling hypertension, can reduce cyclosporin-induced nephrotoxicity (Jackson & Babich 1997). More *severe* gingival changes were observed and the *incidence* of clinically significant gingival overgrowth was found to be higher in patients receiving concomitant treatment with cyclosporin and nifedipine (Slavin & Taylor 1987; Pernu *et al.,* 1993; Thomason *et al.,*1993; Bökenkamp *et al.,* 1994; Rossmann *et al.,* 1994; O'Valle *et al.,* 1995; Darbar *et al.,* 1996; Jackson & Babich, 1997; Garzimo-Demo *et al.,* 1998; Nakayama *et al.,* 1998; Nohl & Ferrari, 1998; Santi & Bral, 1998; Morisaki et al., 2000).

Consistent with these observations Aufricht and co-workers (1997) found that antibiotic treatment of cyclosporin-induced gingival overgrowth in children had only effect with respect to resolution of symptoms consistent with gingival inflammation. They observed no improvement in gingival overgrowth in any of the renal transplant children after metronidazole treatment. The children were however all on concomitant treatment with calcium channel blockers and the synergistic effects of calcium channel blockers and cyclosporin might have outweighed the beneficial effects of metronidazole.

2.5.9.2 Interaction between phenytoin and other anticonvulsant agents

The possible effect of multiple anticonvulsant therapy on the expression of phenytoin-induced gingival overgrowth has been investigated by Maguire and co-workers (1986), who reported an increased incidence of gingival overgrowth in paediatric epileptic patients receiving phenytoin in combination with one or more other anticonvulsants. This is in contrast with the results of a similar study undertaken by Kamali and co-workers (1999) who found that concomitant medication of phenytoin with other anticonvulsants did not lead to an increase in the expression of phenytoin-induced gingival overgrowth in adult epileptics.

The contrast in these findings could possible be explained by the fact that the incidence of gingival overgrowth has been reported to be higher in children and adolescents than in adults (see 2.5.6, p30).

2.6. TREATMENT OF GINGIVAL OVERGROWTH

Significant correlations between the occurrence and/or severity of drug-induced gingival overgrowth and the presence of plaque and calculus accumulation have been reported in numerous studies (see 2.5.7, p31). Bacterial plaque has been causally linked to gingival and periodontal disease and has provided a logical focus for efforts directed at preventing or containing the process of gingival overgrowth. Oral hygiene represents the risk factor most likely to be controlled by the patient, dental hygienist and dentist. Patients must be informed of the tendency for the gingival enlargement to occur in responders. With proper instruction, motivation, assessment and reinforcement, oral hygiene may be effectively addressed by the patient during the course of a systematic approach to periodic and recurring supportive treatment (Hallmon & Rossmann, 1999). For institutionalised patients, such care is sporadic and dependent on others, and consequently often deficient. Approaches may need to be modified in an effort to meet patient needs. This may include the use of antimicrobial mouthrinses, antibiotics and surgical procedures.

2.6.1 Chlorhexidine

Chlorhexidine is a mouthrinse that has been proven to result in significant reduction in plaque accumulation and gingivitis. In a study by Pilatti and Sampaio (1997) statistically significant lower gingival overgrowth was found in a group of Holtzman rats treated with cyclosporin and chlorhexidine than in a group treated with cyclosporin only. This led to the conclusion that if these

studies could be replicated in humans, the application of 0,12% chlorhexidine may be a valuable measure in the management of cyclosporin-induced gingival overgrowth. Santi and Bral (1998) also found the use of chlorhexidine to be beneficial.

2.6.2 Antibiotics

Azithromycin is a semisynthetic macrolide derived from erythromycin which does not modify the pharmacokinetics of other drugs, especially cyclosporin (Amacher *et al.*, 1991). The use of this drug on the treatment of cyclosporin-induced overgrowth has shown to be an effective and safe drug for treating the overgrowth affecting transplant patients (Gomez *et al.*,1997; Pilatti & Sampaio, 1997; Nash & Zaltzman, 1998; Santi & Bral, 1998). Palomar and co-workers (1998) reported a substantial improvement in gum overgrowth and gingival bleeding one week after azithromycin therapy was introduced, with no related side effects.

2.6.3 Surgical procedures

Pocket reduction by periodontal management of excess tissue can be achieved by a gingivectomy (Abitbol & Rosenfeld-Abitbol, 1996; Khocht & Schneider, 1997), laser treatment (Russo, 1997; Mattson *et al.*, 1998) or periodontal flaps (Pilloni *et al.*, 1998).

2.6.4 Substitute drugs

Switching hypertensive therapy to isradipine in hypertensive patients with nifedipine-induced gingival overgrowth (Westbrook *et al.,* 1997) and conversion from cyclosporin to tacrolimus, a new immunosuppressive molecule, (Bader *et al.,* 1998; Busque *et al.,* 1998; Mihatsch *et al.,* 1998; James *et al.*, 2000; Kennedy & Linden, 2000) have been suggested. Avoidance of concomitant treatment with calcium channel blockers, which potentially aggravate this condition, has been recommended (Bökenkamp *et al.,* 1994). However, in some cases treatment cannot be stopped or changed to other drugs because of the severity of patient condition.

The pathogenesis of gingival overgrowth associated with the mentioned three groups of drugs is uncertain and probably multifactorial. Several factors have been implicated such as the dental plaque, a threshold concentration of drug, and an increased activity of fibroblasts. Oral hygiene for controlling plaque and surgical gingivectomy have been used to treat severe cases, but no definite treatment is available. Although changing to other drugs has been proposed, several studies on some of the recently introduced drugs indicate that they give the same side effects (Mihatsch *et al.,* 1998). Gingival overgrowth is probably influenced by a number of factors that need to be investigated more intensively.

CHAPTER 3: AIM OF THE STUDY

From the literature it is clear that numerous studies have been performed to investigate the mechanism by which drugs affect the gingival tissue. Increased proliferation of gingival fibroblasts, a reduction in the degradation of collagen or accumulation of sulphated glycosaminoglycans could be responsible for the increased gingival volume, but it is not known whether the drug influences the metabolism of the gingival cells directly.

In order to investigate the effect of cyclosporin and amlodipine on the growth of fibroblasts and the production of collagen by these cells, this project was undertaken to determine the changes in fibroblast proliferation and the levels of different types of collagen present in fibroblast cell lines derived from normal and overgrown gingival tissue.

The aim of this study was to:

- Clinically differentiate between responder and non-responder transplant patients from the Cardiac Rehabilitation Unit, University of Pretoria (UP).
- Cultivate human gingival fibroblasts from these patients.
- Investigate the effects of cyclosporin and a combination of cyclosporin and amlodipine administered directly to the growth medium on proliferation rates of these cells.
- Investigate the production of collagen types IV, V and VI by these cells, using immuno-fluorescence techniques.

CHAPTER 4: MATERIALS AND METHODS

4.1. SELECTION OF EXPERIMENTAL GROUPS

4.1.1 Patient groups

Detailed examinations of the gingiva in the buccal and lingual segments of the maxilla and mandible of transplant patients was perfomed (Dr. T. Winstanley, Compromised Patient Unit, Oral and Dental Hospital, Pretoria) to determine the presence of gingival overgrowth (see Figure 4.1). Generally, if any enlargement or abnormal overgrowth was obvious in the buccal or lingual gingivae, the patient was classified as a responder. Classification of patients was based on the presence or absence of gingival overgrowth and the severity of overgrowth was not a variant in this study.

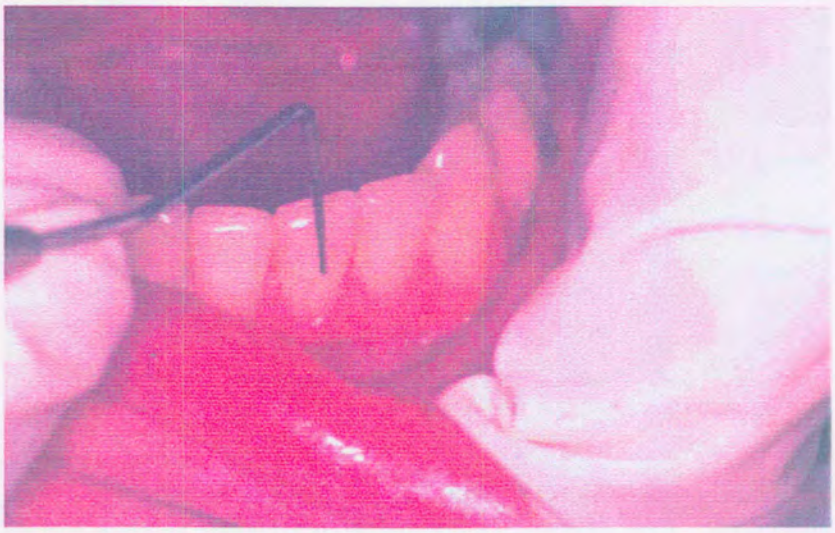

Figure 4.1 Clinical examination of the gingiva for the presence of gingival overgrowth.

After examination for gingival overgrowth, two groups of patients from the Department of Cardiac Rehabilitation, Faculty of Medicine, University of Pretoria, as well as a control group which included healthy individuals, who were not exposed to medicine, were used in this study:

i) Responders: (n=5) transplant patients receiving cyclosporin medication and responding with gingival overgrowth.

ii) Non-responders: (n=5) transplant patients receiving cyclosporin medication but not showing any signs of gingival overgrowth.

iii) Control: (n=5) healthy individuals receiving no medication and with apparently healthy gingiva.

4.1.2 Treatment groups

After the biopsies were grown into cell lines (see 4.2, p42) the fibroblast cell lines of all three groups mentioned in 4.1.1 were treated with the following:

i) Essential Modified Eagle's Media (EMEM) + 10% Fetal Calf Serum (FCS) – control (EMEM).

ii) Cyclosporin: 260μg/ml cyclosporin in EMEM + 10% FCS (CYC).

ii) Cyclosporin + amlodipine: 260μg/ml cyclosporin and 30 μg/l amlodipine in EMEM + 10% FCS (CA) (Figure 4.2)

a. b.

Figure 4.2 Drugs used to treat the different cell lines: (a) Cyclosporin (b) Amlodipine

4.2. DEVELOPMENT AND MAINTENANCE OF CELL LINES

From all the biopsies taken from the control group, responder group and the non-responder group, fibroblast lines were cultivated. The cultivation procedures of Botha (1995) were followed with minor modifications. Media and reagents used for cultivation of fibroblasts and the immunohistological detection of collagen is given in Table 4.1.

4.2.1 Biopsy collection and preparation

After appropriate approval and patient consent (Addendum A) a small biopsy ($2mm^3$) was removed from the gingival tissue of individuals of each group during periodontal treatment (see Figure 4.3). Dr. T. Winstanley performed all biopsies.

Table 4.1 Media and reagents that were used for the cultivation of explant cultures from gingival tissue and immunohistological detection of collagen.

Media	Supplier
EMEM with Penstrep (1% Penicillin and 1% Streptomycin)	National Institute of Virology (NIV) Private Bag X4 Sandringham 2131
Fungizone	Highveld Biological (Pty) Ltd. P.O.Box 488, Kelvin, 2054
Penicillin & Streptomycin sulphate (0,1 mg/ml)	Highveld Biological (Pty) Ltd. P.O.Box 488, Kelvin, 2054
Fetal calf cerum (FCS)	Sterilab Services P.O.Box 2021, Kempton Park, 1620
Ultra Culture	Sterilab Services P.O.Box 2021, Kempton Park, 1620
Trypsin-EDTA	National Institute of Virology (NIV) PVT Bag X 4 Sandringham 2131
Phosphate Buffered Saline (PBS –Buffer)	Research Centre for Stomatology University of Pretoria. Lab Production:144,00mg/l KH_2PO_4; 9,000mg/l NaCl; 795,0mg/l Na_2HPO; pH 7,6
Tissue culture flasks 25 cm^3; 75 cm^3	Sterilab Services P.O.Box 2021, Kempton Park, 1620
Disposable sterile pipettes – 5ml and 10ml	Sterilab Services P.O.Box 2021, Kempton Park, 1620
Corning cell wells – 24 and 96	Sterilab Services P.O.Box 2021, Kempton Park, 1620
Trypan Blue	Sigma Aldrich (Pty)Ltd P.O.Box 12202, Vorna Valley, 1686
Neubauer counting chambers	Sigma Aldrich (Pty)Ltd P.O.Box 12202, Vorna Valley, 1686
Finnpipette 200-1000 ul	AEC – Amersham (Pty)Ltd. P.O.Box 1596, Kelvin, 2054
Gilson Pipetman P100	Labotec P.O.Box 6553, Halfway House, 1685
Collagen Types IV, V, VI and Mouse antibody anti human; Fluorecein Anti Mouse IgG	Separations (Pty)Ltd. P.O.Box 4181, Randburg
Human Collagen Type IV	Sigma Aldrich (Pty)Ltd P.O.Box 12202, Vorna Valley, 1686

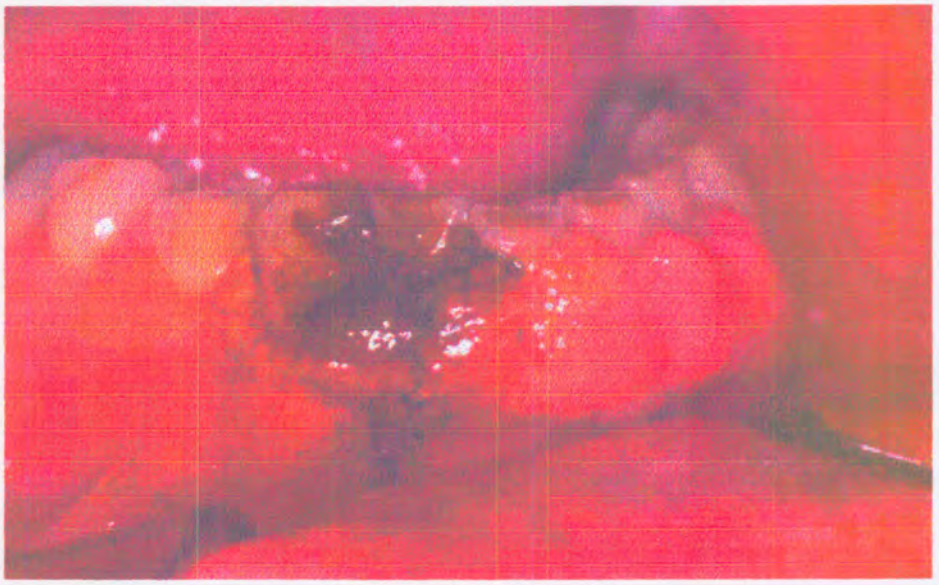

Figure 4.3 During periodontal treatment a small biopsy from the gingival tissue was removed.

Within 24h the biopsies were pretreated with a PBS Penstrep mixture to remove all excess blood and possible bacterial and fungal contaminants. The biopsies were placed in 9cm sterile petridishes and excess PBS mixture removed using a sterile pipette. The tissue was then cut with a sterile scalpel blade and mashed into smaller cubes (see Figure 4.4).

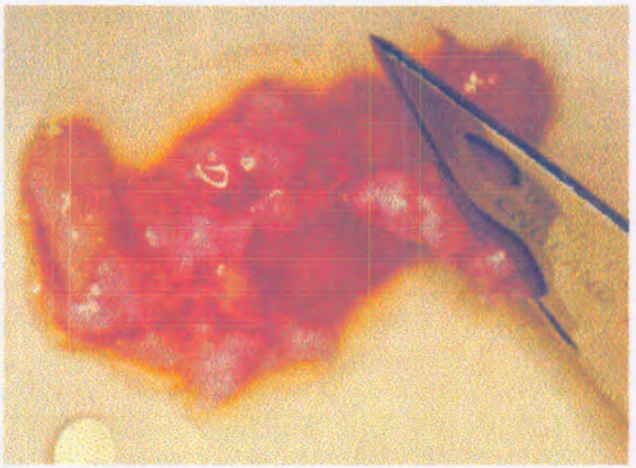

Figure 4.4 Gingival tissue was cut and mashed into smaller cubes.

For primary outgrowth 0,5 ml Ultra Culture (see Table 4.1, p43) was used to seed the mashed tissue into FCS pretreated 25T flasks. Pretreatment was done by pipetting 0,5 ml FCS into the tissue culture flasks and then rotating the flasks so that the serum was distributed in a homogeneous film over the surface. These surfaces were left to stand for at least 1 hour to improve the stickiness of the surface which would enhance tissue attachment that is needed for outgrowth of fibroblasts.

Cultivation and maintenance of cell cultures were done at 37°C in 99% relative humidity and an atmosphere of 5% CO_2 and 95% air. For all routine cell culture procedures sterile disposable plastic-ware was used and all procedures were done in a Fibatron laminar flow head[1] at a flow speed of 100 Pascals to ensure sterility.

After 5 days the samples were examined for outgrowth using an inverted microscope (Zeiss Axiovert 25[2]) (100x) and without disturbing the tissue fragments, 2 ml of Ultra Culture was carefully added to the flasks. The media were hereafter changed at twice week intervals. Proliferating cultures were gradually weaned from Ultra Culture and maintained in EMEM with 0,1mg/ml Penicillin 0,1mg/ml Streptomycin supplemented with 10% heat inactivated FCS and 0,25 ug/ml fungizone until confluency was reached (see Table 4.1, p43).

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1 Fibatron, P.O.Box 4988, Johannesburg, 2000, RSA.
2 Carl Zeiss (PTY) LTD, P.O. Box 3003, Randburg, 2125, RSA.

4.2.2 Passage of explant primary cells

The cells from confluent cultures were detached from the tissue culture plastic using a mixture of 0,5% trypsin and 0,2% EDTA (ethylene-diamine-tetra–acetic-acid). Trypsinisation was carried out by the removal of the media and rinsing the flask twice with 1 ml PBS. 5 ml Trypsin-EDTA was then pipetted onto cells and the flask rinsed with the mixture. The flasks were then incubated for 2-5 minutes at 37°C. Observations using an inverted microscope were constantly made to confirm the detachment of the cells. As soon as the cells were completely detached the trypsinisation reaction was ended by adding 5 ml EMEM + 10% FCS. Cells were then agitated to obtain a single cell suspension by pipetting of the suspension. Of this cell suspension 4ml was transferred to 75 cm^2 tissue culture flasks containing 10ml of EMEM + 10% FCS (passage 1) and 1 ml was kept in 25 cm^2 culture flasks as precaution.

4.2.3 Multiplication of cells in cell lines

For the multiplication of cells, EMEM and the same culture conditions as for primary cultivation were used. Cells that reached confluence in a tissue culture flask were subcultured into four new flasks (1:4).

4.2.4 Maintenance of cells

For all normal tissue culture procedures and the duration of the experimental procedures, cell lines were maintained in EMEM. However, for longer storage periods cells in their logarithmic growth phase were suspended in EMEM with high glucose and pyruvate and with L glutamine. The media was supplemented with 10% heat inactivated FCS, 1% of a non-essential amino-acid mixture, and 10 % Dimethyl Sulfoxide (DMSO)(Botha 1995). No antibiotics were added to the media. The suspension of cells was divided into 2ml aliquots and differentially frozen as indicated in Table 4.2. Cells were eventually stored in liquid nitrogen (-196°C).

Table 4.2 Differential freezing of cells for long term storage in liquid nitrogen.

PROCESS	TEMPERATURE	TIME
Refrigeration	4°C	12 h
Freezing (laboratory freezer)	-20°C	12 h
Freezing (-70°C freezer)	-70°C	12 h
Liquid nitrogen	-196°C	Long term storage

When cell lines needed to be activated a tube with frozen cells was removed from the liquid nitrogen and thawed quickly in a 60°C waterbath. This thawing process was achieved by sequential dipping of the tip of the tube into the

waterbath for very short periods. As soon as the frozen medium was visually thawed, the DMSO containing media were removed by centrifugation and by washing the cells twice with 2ml phosphate buffered saline. Cells were then resuspended in fresh EMEM, incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air and normal procedures for cultivation of cell cultures were hereafter followed.

4.3. IMPLEMENTATION OF EVALUATION PROCEDURES FOR FIBROBLAST REACTION TOWARDS CYCLOSPORIN AND CYCLOSPORIN + AMLODIPINE.

4.3.1 Preparation of Cyclosporin:

Neural Cyclosporin 25 mg (Sandoz[3]) was obtained from the Heart Transplant Clinic. The mean of the serum cyclosporin concentration was calculated after a pool of the values from the patients taking part in the experiment was obtained from the Department of Microbiology. The mean cyclosporin serum concentration was 260,27 µg/ml (TDX monoclonal Antibody Test). Therefor a concentration of 260µg/ml cyclosporin was made in EMEM + 10% FCS.

[3] Supplied by Novartis, 72 Still Road, Spartan, 1600, South Africa

4.3.2 Preparation of Amlodipine:

A combination concentration of 260µg/ml cyclosporin and 30 µg/l amlodipine[4] (SA Medicines Formulary, 2000) was made in EMEM + 10% FCS.

4.3.3 Standardisation of cell cultures in cell wells.

Tissue culture treated polystyrene 24-well with lid cell wells[5] were used to perform the experiment. Six confluent flasks of each responder (n=5), non-responder (n=5) and control group (n=5) were cultured. The passages varied between 3 – 6.

The confluent flasks were trypsinised with 5 ml 0.5% Trypsin and 0,2% EDTA mixture and calibrated single cell suspensions were prepared ([cell] = 1,5 - 3 x 10^4 cells per ml) in:

i) EMEM

ii) 260µg/ml cyclosporin in EMEM + 10% FCS

iii) 260µg/ml cyclosporin and 30 µg/l amlodipine in EMEM + 10% FCS.

From each of the three cell suspensions from every patient cells were seeded in cell wells by pipetting 1 ml of cell suspension in the cell wells. Special care was

[4] Supplied by Pfizer, Southern Park, 102 Rivonia road, Sandton, South Africa

[5] Sterilab Services cc., P.O.Box 2021, Kempton Park, 1620, RSA.

taken to make sure that a single cell suspension and even distribution of cells in all the cell wells was obtained during the seeding process.

4.3.4 Counting procedures

Neubauer counting chambers were used in this study for the enumeration of cell numbers in cell suspensions (see Figure 4.5). In all instances the trypan blue exclusion method was used for the determination of viable cells (Botha, 1995). At no stage was a cell suspension that was mixed with trypan blue allowed to stand for longer than 5 minutes, since extended periods of exposure of cells to trypan blue can result in the uptake of dye by both viable and non-viable cells, resulting in an inability to distinguish between dead and viable cells.

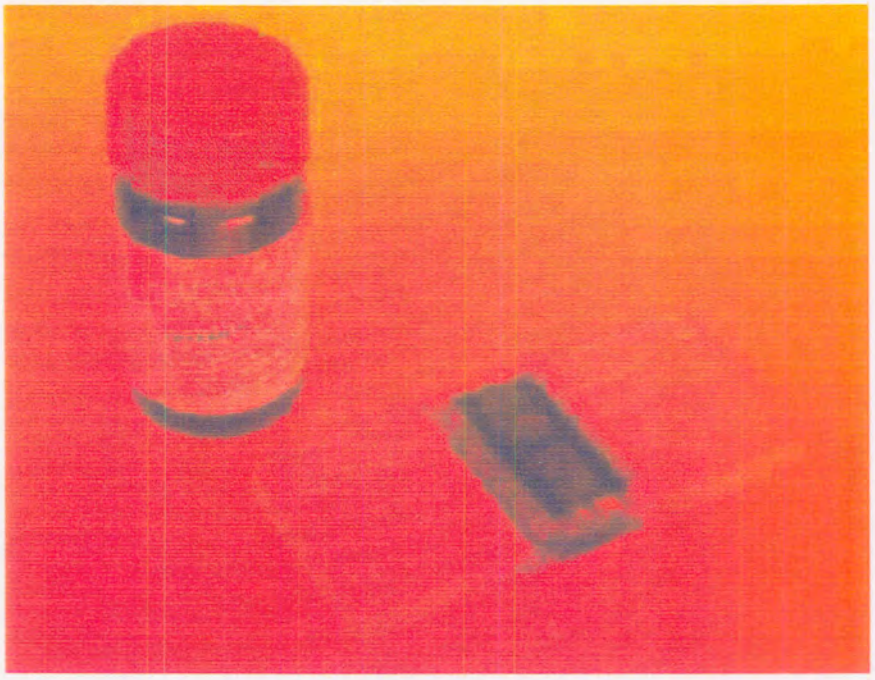

Figure 4.5 Trypan Blue and a Neubauer counting chamber

One well of the cell-well plate was examined by inverted microscope to make sure that the cells were healthy. The specific well was treated as follows:
The media was removed with a pipette, the cell well was rinsed twice with PBS, 0,5ml Trips EDTA mixture was added and incubated for ± 5 min in 37°C to allow detachment of cells. Detachment was confirmed by examination with the inverted microscope. The trypsination reaction was stopped by adding 0,5ml 10% EMEM. Hereafter the cell suspension was transferred to 1ml of 0,4% Trypan blue. The cell suspension was then loaded in a Neubauer counting chamber and counted. Twenty fields were counted for each determination.

The cell concentrations of the different series of wells were determined over a period of ten weeks twice weekly. During this time the media of each series were changed once a week to maintain growth.

4.3.5 Cell growth curves

The cell count after 24h (t_0) was used as standardised value for the onset of each growth curve. Average counts per time intervals were calculated for the 20 fields counted. Percentage increase in cell numbers was determined by using t_0 as reference point (Ms. S. Rothman[6] (UNISA, SA) and growth curves were compiled using these results.

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[6] Mrs. Synthia Rothman, Senior Lecturer, University of South Africa, P.O. Box 392, Muckleneukrant,Pretoria, RSA.

4.3.6 Statistical analysis

Statistical analysis of data (tested by ANOVA) to determine maximum values over time for each group was performed by Dr. P Becker[7] (MRC, SA).

4.4 DETERMINATION OF COLLAGEN

4.4.1 Dilution of antibodies

Collagen types IV, V and VI mouse antibody anti-human antibodies[8] were prepared according to product specifications (Addendum B). Different dilutions were experimented with but a 1:50 dilution seemed to be the most effective for this experiment.

The secondary antibody and marker fluorecein-antimouse IgG[8] was also used in a 1:50 dilution. The recommended 5% goat serum in 50mm phosphate-buffered saline was replaced with 5% fetal calf serum after communication with Biomedia via Seperations Scientific. The positive control Collagen type IV 1mg/ml was reconstituted in 5 ml 0,25% acetic acid, distilled water and alloquoted until needed.

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7 Dr. Piet Becker, Medical Research Council, Pretoria, RSA.
8 Supplied by Separations (PTY)LTD, P.O.Box 4181, Randburg, RSA.

4.4.2 Cell well preparation

For determination of collagen presence polystyrene tissue culture 96-well Corning cell wells[9] were seeded with cell suspensions (cultured as explained in 4.3) of responder (n=3), non-responder (n=5) and control (n=1) groups. The cell wells were examined by using the inverted microscope to ensure even cell distribution and the cell wells were incubated in 37°C, 5% CO_2 and 95% air.

4.4.3 Method of fixing cells in cell wells

Cells were fixated after 2 week and 8 week intervals. Wells were washed with 2 x 1ml PBS using a micropipette and then washed once with distilled water to remove any salt crystals. The cells were fixed using 0,05 ml ETOH 96% at -20°C for 5 minutes. Wells were dried and stored at 4°C. Type IV collagen was fixed as positive control by adding 10µl in the control well, rotating to ensure even distribution over the bottom surface of the well and allowing to dry at 37°C in a fan incubator.

Cell wells were removed from the 4°C and rinsed once with PBS for 5 minutes. The wells were then dried using the capillary forces of filter paper. 30µl Mouse Anti Human Collagen types IV, V and VI were added to the wells. PBS was

[9] Supplied by Sterilab Services P.O.Box 2021, Kempton Park, 1620

used as negative control. Wells were then incubated at 37°C in a sealed plastic container with moist tissue paper for 18 hours.

After 18 hours wells were rinsed twice and washed for 3 x 10 minutes in PBS to remove the excess antibodies. Secondary antibodies Anti Mouse Fluoresceinisothiocyanate (FITC) were added (30µl) and wells were incubated at 37°C for 18 hours. The cell wells were examined using an Axiovert HBO50 Halogen lamp[10] for fluorescence.

The reliability of the test was confirmed by the presence of fluorescence of the positive control and the absolute absence of fluorescence of the negative control.

Quantification of different collagen types was measured using a Flexible Image Processing System[11] (FIPS: CSIR). The image observed on the microscope was transferred to a computer and for quantification of the collagen, the fluoresced surface was traced and the total area size and the percentage cover were calculated and tabulated.

[10] Carl Zeiss (PTY) LTD, P.O. Box 3003, Randburg, 2125, RSA.

[11] Counsil for Scientific and Industrial Research, Meiring Naude Street, Brummeria, Pretoria

CHAPTER 5. RESULTS

5.1. CLINICAL EXAMINATION

The detailed clinical examinations of the gingivae of transplant patients revealed patients with apparent normal gingivae - **non-responders**, and patients with clinically obvious qualitative changes of varying degree in the gingivae – **responders** (see Figure 5.1)

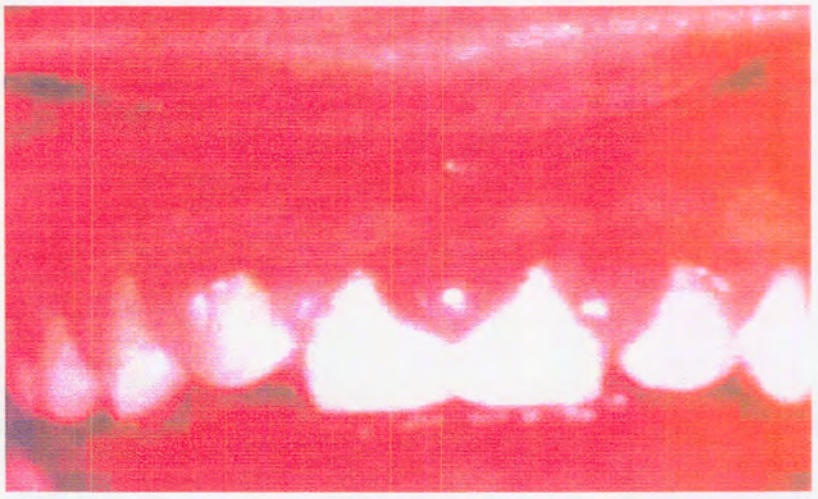

Figure 5.1 The gingivae of a patient who has been identified as a responder.

5.2 CULTIVATION AND MAINTENANCE OF CELL CULTURES

5.2.1 Cultivation procedure

During cultivation of the fibroblasts it was important to take special care regarding the sterility of the procedures, since it was frequently observed that

highly resistant fungi contaminated the cultures. Contamination from bacteria was efficiently suppressed by the addition of Penstrep. Explant cultures were obtained from all the biopsies taken, therefore the success rate of cultivation was 100%.

5.2.2 Maintenance of cell cultures

Maintenance and multipication of cells were effective with EMEM as media. Fibroblast cells were effectively stored in media containing DMSO in liquid nitrogen after differential freezing of the cells (see Table 4.2, p47). Proliferation of cells after storage in liquid nitrogen was 100%.

5.3 IMPLEMENTATION OF EVALUATION PROCEDURES FOR FIBROBLAST REACTION TOWARDS CYCLOSPORIN AND AMLODIPINE

5.3.1 Standardisation of cell cultures

It was at all times possible to multiply cells and standardise cell cultures when the correct media and conditions for cell cultures were used. From standardised cultures, cells that were in an active mitotic state were continuously obtained for the inoculation of experiments.

5.3.2 Preparation of single cell suspensions and cell counts

A summary of the results obtained after preparation of single cell suspension and the determining of cell concentrations of the different series of wells over a period of ten weeks is given in table C.1 (Addendum C).

5.3.3 Cell growth curves

A summary of the percentage increase in cell numbers is given in table D.1 and the growth curves of each individual cell line are given in figures D.1 – D.15 (Addendum D).

Figures 5.2 – 5.4 give the mean percentage increase in cells for the control, responder, and non-responder groups respectively. During the cultivation period the incubation of all cell lines showed kinetics typical of fibroblasts with an initial log phase followed by a plateau.

In the first six weeks of growth there was no significant difference in proliferation of cells lines between the control cell lines in the presence of EMEM and CA (Figure 5.2). During weeks 7-8 the control cell line treated with CA reached a maximum percentage increase in cells of 1325% whereas the cell line treated with EMEM reached a maximum of 1118%. The control cell line treated with CYC showed a considerably lower growth rate than the other two groups of

cells peaking at 721%. During weeks 8 to 10 there was a marked decline in cell growth in the presence of CA.

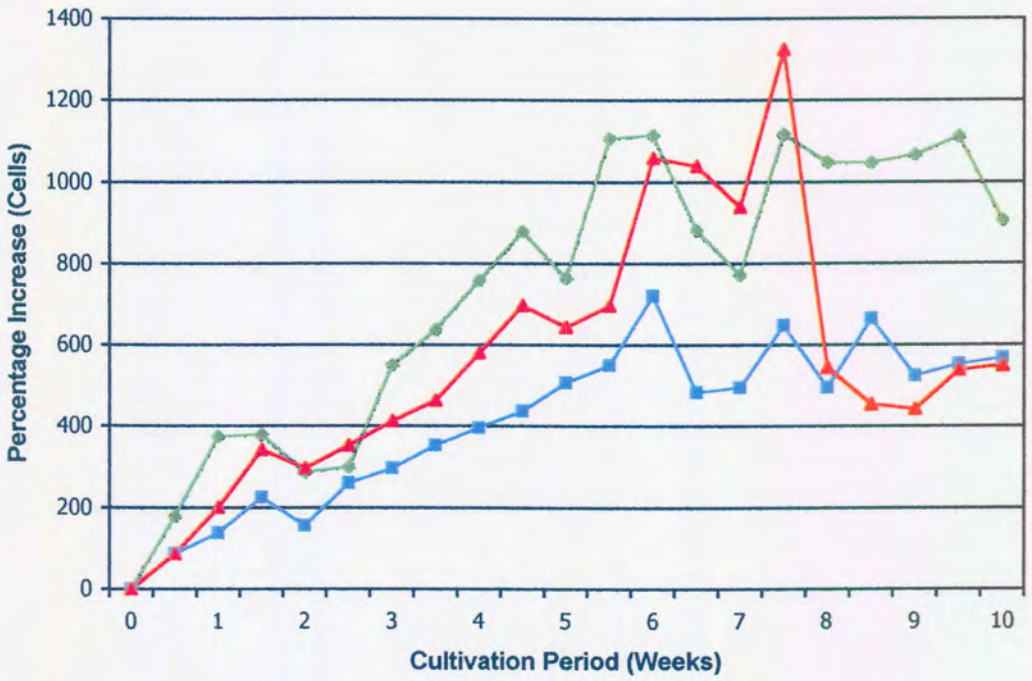

Figure 5.2 Mean percentage increase in human gingival fibroblasts from the control group when cultured in EMEM (◆); EMEM + 260µg/ml Cyclosporin (■) and EMEM + 260µg/ml Cyclosporin + 30µg/l Amlodipine (▲).

The growth curves for the responder cell lines (Figure 5.3) showed a high proliferation and growth rate in the presence of CA especially during week 6 to 8. The maximum percentage increase obtained by these cell lines was 1650%. There was no significant difference in proliferation of cells lines between the responder cells in the presence of CYC and EMEM (Peak values of 1352% and

1224% respectively). From week 8 the responder cell lines showed a marked decline in cell numbers for all three treatments.

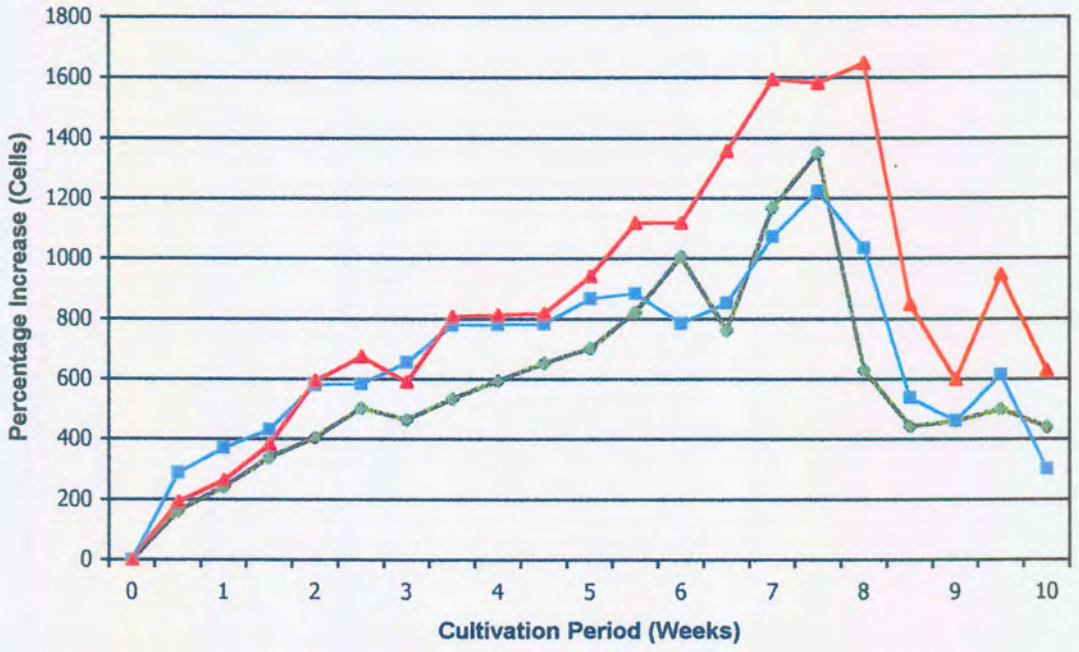

Figure 5.3 Mean percentage increase in human gingival fibroblasts from the responder group when cultured in EMEM (◆); EMEM + 260µg/ml Cyclosporin (■) and EMEM + 260µg/ml Cyclosporin + 30µg/l Amlodipine (▲).

The non-responder cell lines (Figure 5.4) grew well in all media but were inhibited by the addition of CYC and CA. The maximum values in mean percentage increase for all three cell lines were in general lower than the values obtained by the control and responder groups. The non reponder cell lines grown in EMEM showed the highest mean percentage increase (820%) followed by the CYC treatment (574%) and CA treatment (470%).

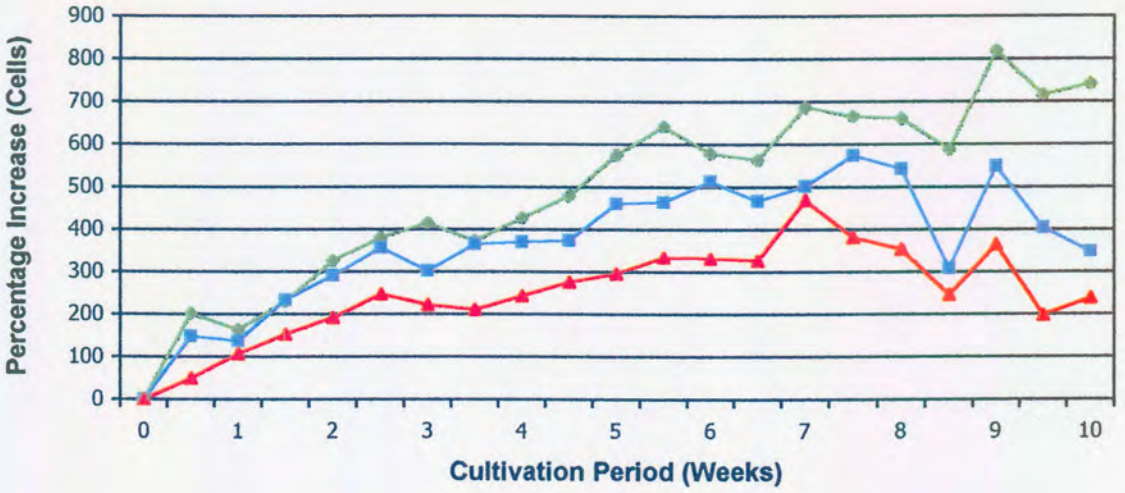

Figure 5.4 Mean percentage increase in human gingival fibroblasts from the non-responder group when cultured in EMEM (◆); EMEM + 260µg/ml Cyclosporin (■) and EMEM + 260µg/ml Cyclosporin + 30µg/l Amlodipine (▲).

Figure 5.5 gives a comparison of the mean growth rates of control, responder and non-responder cell lines exposed to CYC. The results show a significant differerence between the maximum percentage increase (1224%) for responder cell lines compared to non responder cells (574%). The control cell lines in the presence of CY reached a maximum percentage increase of 721%.

Figure 5.6 gives a comparison of the mean growth rates of control, responder and non-responder cell lines exposed to CA. Once again a stimulation of growth was observed for the responder cell lines (1650%). With CA stimulation of growth was also observed for the control group (1325%). The non-responder cell lines were inhibited in the presence of CA (470%).

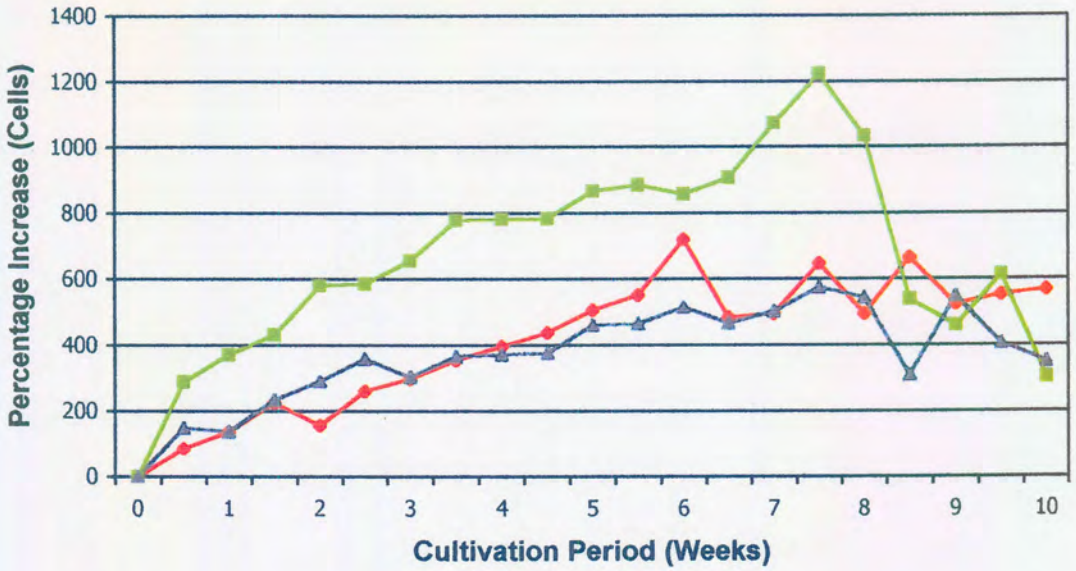

Figure 5.5 Mean percentage increase in human gingival fibroblasts from the control (◆), responder (■) and non-responder (▲) groups when cultured EMEM + 260µg/ml Cyclosporin.

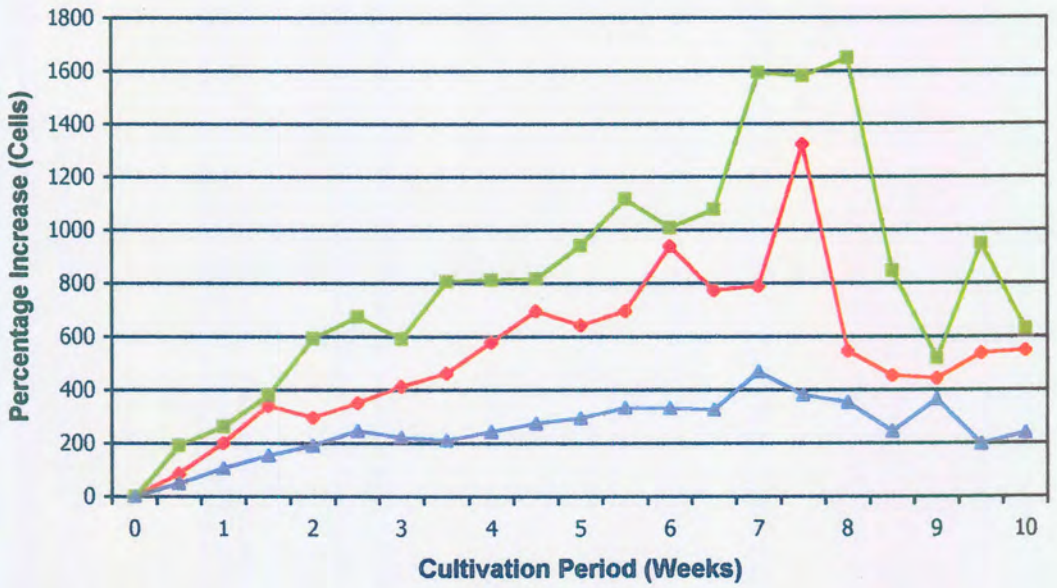

Figure 5.6 Mean percentage increase in human gingival fibroblasts from the control (◆), responder (■) and non-responder (▲) groups when cultured in EMEM + 260µg/ml Cyclosporin + 30µg/l Amlodipine.

5.3.4 Statistical analysis

Table 5.1 shows the results of statistical determination of maximum percentage values for the different study groups and treatments. These results confirmed the tendencies shown in the growth curves.

Table 5.1 Mean maximum values determined for the different study groups.

	EMEM	CYC	CA
CONTROL	1435.4%	905.9%	1260%
RESPONDERS	1197.7%	1256.9%	1885.7%
NON-RESPONDERS	1054.3%	801.5%	641.3%

These results confirm an overall higher proliferation rate for responder cell lines (compared to the non responders and control groups) grown in EMEM and exposed to CYC and especially CA. The control cell lines grew well in all three treatments and the non-responder cell lines were inhibited by CYC and CA.

5.3.5 Results of immunofluorescence determination of different types of collagen.

The results obtained by the Flexible Image Processing System (FIPS) and graphs representing the mean percentage coverage by each type of collagen (IV, V and VI) are given in Addendum E. Table 5.2 gives the percentage

coverage by each type of collagen (Types IV, V and VI) for the control group, responders and non-responders.

Table 5.2 Percentage coverage by different collagent types for the different patient groups.

	Collagen type IV (%)			Collagen type V(%)			Collagen type VI(%)		
	EMEM	**CYC**	**CA**	**EMEM**	**CYC**	**CA**	**EMEM**	**CYC**	**CA**
C	0	0	0	0	0	0	5.13	3.54	6.49
Mean	**0**	**0**	**0**	**0**	**0**	**0**	**5.13**	**3.54**	**6.49**
R1	0	0	0.65	0	0	0	2.09	6.30	7.45
R2	1.62	1.51	0	0	0.32	0.96	4.85	8.62	7.84
R3	0	0	0	0	0.57	3.68	5.02	7.08	8.00
Mean	**0.54**	**0.5**	**0.22**	**0**	**0.3**	**1.55**	**3.99**	**7.33**	**7.76**
NR1	0	1.92	0	0	0.77	0.69	2.48	8.08	3.52
NR2	0	0	0	0	0	0.43	3.04	10.92	2.87
NR3	0	0.50	0	0	0	0.20	0.97	7.30	3.47
NR4	0	1.19	0	0	0	0	2.36	2.89	7.27
NR5	0	0	0	0	0	0	3.45	5.3	5.58
Mean	**0**	**0.72**	**0**	**0**	**0.15**	**0.26**	**2.46**	**6.90**	**4.54**

As can be seen from Table 5.2 there was an absence of collagen types IV and V in the control group. Collagen type VI production in the control group was highest when the cell line was treated with CA (6,49%). The reponder cell lines showed very low percentage collagen type IV and collagen type V coverage (values not exceeding 1,55%) but collagen type VI was present in greater amounts when responder cell lines were grown in EMEM (3,99%), CYC (7,33%) and CA (7,76%). The non-reponder cell lines also showed very low percentage collagen type IV and collagen type V coverage (values not exceeding 0,72%) and collagen type VI in greater amounts when non-responder cell lines were grown in EMEM (2,46%), CYC (6,9%) and CA (4,54%).

CHAPTER 6. DISCUSSION

Drug-induced gingival overgrowth is a condition characterised by formation of tissue pockets commencing in the interdental papillary tissue and extending coronally to the occlusal surface. It is an aesthetically disfiguring condition that can cause discomfort and functional problems during speech and mastication (Jackson & Babich, 1997). Of the predisposing factors associated with this disfiguring and functionally compromising overgrowth of gingival tissue, selective anticonvulsants, calcium channel blockers and immunosupressants, especially cyclosporin, have generated a large amount of investigative attention in the dental and medical community.

6.1 PATIENT DIFFERENTIATION

Two categories of patients have been identified for drug-induced gingival overgrowth namely responders where an enlargement of the gingiva develops and non-responders where such lesions are absent (Chee & Jansen, 1994). We have been able to differentiate between responder patients who had clinically obvious qualitative changes in the gingiva and non-responder patients with apparent normal gingivae. The clinical features of responder patients agreed with the features described by other investigators. In severe cases the enlargement was found to be more pronounced on the labial gingiva of the lower anterior teeth as found by Ramon and co-workers (1984) and Van der

Wall and co-workers (1985). In the most severe cases the gingival tissue appeared lobulated and the crowns of the teeth were partially obscured.

6.2 FIBROBLAST REACTION TOWARDS CYCLOSPORIN AND AMLODIPINE

The etiology of drug-induced gingival overgrowth has not been determined exactly and there have been several mechanisms proposed. One theory suggests that there is a direct effect on functionally heterogeneous subpopulations of fibroblasts. Different subgroups of fibroblasts have distinct characteristics in terms of cell proliferation rate and collagen and protein production and may respond differently to chemical agents.

Increased proliferation rates of fibroblasts isolated from hyperplastic gingival tissue have been shown in fibroblastic populations exposed to cyclosprin (Zebrowski *et al.*,1986; Jacobs *et al.*,1990; Mariani *et al.*,1993; Zebrowski *et al.*,1994 Tipton *et al.*, 1997;), nifedipine (Barak *et al.*,1987; Fuji *et al.*, 1994) and phenytoin (Sasaki *et al.*, 1998). These investigators demonstrated that cultures of gingival fibroblasts, from patients who reacted to any of these drugs (responders), tended to show higher proliferation rates than those from non-reactive patients. McKevitt and Irwin, (1995) showed that at both low and high fetal calf serum levels, cells from responder overgrown tissue grew more quickly and to higher saturation cell densities than the non-responder lines. Our results confirmed high proliferation rates for responder cell lines in the presence of EMEM, CYC and especially CA. In many of the cell lines cultivated during the

study period, a sharp decline in numbers was found during the last two weeks of cultivation. The cell lines which reached very high proliferation rates were affected most (control cell lines exposed to CA, responder cell lines in all three growth media). The limited avaliable space and high cell densities could explain the decline in cell numbers due to unfavourable growth conditions.

The non-responder cell lines showed a decrease in proliferation rates and growth seemed to have been inhibited by the presence of CYC and CA to the media. Evidence supporting the cell selection model has been provided by this study and it is clear that the gingival tissue from responder individuals have more susceptible subpopulations of fibroblasts to certain drugs.

6.3 COLLAGEN PRODUCTION

It can also be speculated that some genetically-predetermined drug-sensitive subpopulations of gingival fibroblasts produce high amounts of collagen (high activity), and others do not produce significant amounts of collagen (low activity). High activity fibroblasts in the presence of a certain threshold concentration of a drug may become 'sensitized' by the drug and there may be a subsequent increase in collagen production (Hassell & Cooper 1980).

Narayanan and co-workers (1988) reported that in two out of three instances, phenytoin-induced fibroblasts produced more collagen than cells from normal control, and had higher levels of collagen mRNA. Hassell and co-workers

(1976) demonstrated that fibroblasts derived from hyperplastic tissue exhibited a level of protein synthetic activity approximately twice that of comparable cells obtained from normal control individuals. Benveniste and Bitar (1980) reported the stimulatory effects of phenytoin on protein and mRNA biosynthesis in cultured human gingival fibroblasts. Increased total protein and collagen production was shown after incubation of gingival fibroblasts in the presence all three drug groups (Wysocki *et al.,* 1983; Zebrowski *et al.,* 1986; Schincaglia *et al.,* 1992, Lucas *et al.,* 1985; Tipton & Dabbous 1993;Narayanan & Page, 1983; Vernillo & Schwartz, 1987; Johnston *et al.,* 1990; Hassell & Gilbert, 1993).

Results of this study are consistent with these findings as the responder cell lines produced quantitatively more collagen type IV, V and VI when compared to the non-responder cell lines. As the production of collagen IV was so low, it was not possible to compare the difference in percentage production for this type of collagen. The responder cells produced 33-72% more of type V collagen and 4-26% more of collagen type VI compared to the non-responder cells. This is in agreement with the study of Fujii and co-workers (1994) who showed cells from nifedipine reactive patients to give 9,6-55,7% greater collagen synthesis rates.

Narayanan and Page (1983) and Bonnaure-Mallet and co-workers (1995) found an abnormal ratio of collagen types I:III. Narayanan and Page (1983) also demonstrated an increase in type V collagen in enlarged gingiva as was confirmed by our results. The *in vitro* studies of Schincaglia and co-workers

(1992) have shown that cyclosporin caused a specific rise in the level of type I procollagen. Due to funds restrictions and availability of collagen types, we did not test for collagen type I, and further investigations are required.

Immunohistological investigations of Bonnaure-Mallet and co-workers (1995) showed a comparatively higher percentage of area occupied by collagen type IV collagen in cyclosporin-induced overgrowth. This is in contrast with our findings as collagen IV production was very low or not detectable by the method used.

Romanos and co-workers, (1993a; 1993b) were able to demonstrate the localization of collagen types I, III, IV, V, VI and VII as well as the glycoprotein fibronectin in nifedipine-induced gingival overgrowth. However, their patient group consisted of responder-patients only and no comparison was therefore made between the production of the different types of collagen by responder and non-responder patients.

6.4 MODELS TO EXPLAIN CELL PROLIFERATION AND COLLAGEN PRODUCTION.

The results of this study show that CA addition to the growth medium of the 'non-responder' cells inhibited cell proliferation. The production of collagen type VI by the non-responder cells was also lower in the presence of CA. It is therefor evident that the addition of a calcium channel blocker to cyclosporin

have an inhibitory influence on the non-reponder cells. By accepting the cell selection model, it could be hypothesised that cyclosporin, and especially with the addition of amlodipine, has a cytotoxic effect on subpopulations of non-reponder cells. The growth and proliferation and the production of collagen is inhibited by the addition of drugs.

Proliferation rates of 'responder' fibroblasts were found to be higher with addition of CA compared to the addition of CYC only. It has been suggested that a certain threshold exist for susceptibility of fibroblasts to the effects of cyclosporin. Some strains of human fibroblasts respond to lower concentrations of cyclosporin, while other strains respond only at higher concentrations. Concomitant treatment with calcium channel blockers could lower the threshold of individual sensitivity of responder gingival fibroblasts.

Phenytoin selectively depresses the motor cortex of the central nervous system and it is believed to mediate this action by stabilising neuronal excitation by blocking or interfering with calcium influx across cell membranes (Leppik, 1990; Rees, 1993).

Calcium antagonists suppress calcium influx through plasma membrane channels in cardiac tissue, vascular smooth muscle and other excitable cells. Thus, the decrease in intracellular calcium concentration leads to characteristic changes of affected tissues (Nyska et al., 1994)

The mechanism of cyclosporin action relates to the inhibition of the production and the release of interleukin 2, ultimately interfering with cellular calcium influx and humoral immune responses (Long, 1984).

Another theory therefor proposes that divalent cations such as Ca^{2+} can act as agents that promote phagocytosis. The plasma membrane and subplasmalemmal zone of phagocytic cells contain Ca^{2+} and Mg^{2+} dependent ATP-ase activity. Thus, it may be significant that the drugs that induce gingival overgrowth may have an inhibitory influence on Ca^{2+} ion passage across cell membranes or may interfere with the interaction of intracellular ionic calcium leading to greater synthesis of collagen and less phagocytosis (McGaw & Porter 1988).

CHAPTER 7: CONCLUSIONS AND RECOMMENDATIONS

Three different groups of drugs have been associated with the occurrence of gingival overgrowth in susceptible individuals namely anticonvulsants, cyclosporin and calcium channel blockers. Despite their pharmacological diversity, these drugs have a similar mechanism of action at the cellular level, where they inhibit intracellular calcium ion influx. Therefore, the action of these various drugs on calcium flux may prove to be the key to understanding the unwanted side effect upon the gingival connective tissue.

Histological appearance of the gingival overgrowth are similar for all three drug-induced overgrowths such as an increase in the number of fibroblasts and extracellular ground substance. There seems to be a relationship between plaque, gingival inflammation and drug-induced gingival overgrowth.

Prevention and treatment of overgrown gingival tissue begins with good oral hygiene practices and many patients respond favourable to non-surgical treatment of the condition. A significant number however require surgical removal of the overgrown tissues. Further studies of overgrown tissue should provide a more thorough understanding of the pathogenesis of this unwanted side effect and this is essential to devise appropriate regimens for its prevention and treatment.

The various investigations into the pathogenesis of drug-induced gingival overgrowth suggests that it is multifactorial and that genetic factors (fibroblast heterogeneity), drug variables, plaque-induced inflammatory changes and growth factors seem to be significant factors in the expression of these gingival changes.

The treatment of choice for drug-induced gingival overgrowth would be alternative drug therapy. This is not realistic when the systemic protective effects of the drugs are more important than the local unwanted side-effect. For such cases the overgrowth has to be accepted, and treated when severe enough to cause aesthetic and functional problems.

Because of the large discrepancy in the prevalence range of gingival overgrowth reported in the literature, further investigations are warranted. Many patients may be at risk of developing or being affected by gingival overgrowth practitioners should be aware that patients on these drugs might be susceptible to oral complications. Guidelines must be designed for both dentists and physicians for early identification

Our study will be extended to investigate other aspects of this side effect, such as quantification of collagen production by fibroblasts, immunology and the involvement of genetic factors in the gingival response of only certain patients to drugs.

CHAPTER 8: LITERATURE

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Addendum A: Let

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Fakulteit Tandheelkunde

1 Maart 1996

Geagte Pasiënt

NAVORSINGSPROJEK OOR HIPERPLASIE VAN GINGIVALE WEEFSEL
TOESTEMMING VIR DIE NEEM VAN WEEFSELBIOPSIE

Personeel van die Sentrum vir Stomatologiese Navorsing is huidiglik besig met 'n projek wat handel oor geneesmiddel geïnduseerde oorgroei van tandvleisweefsel. Daar is veral drie groepe pasiënte wat deur hierdie genoemde oorgroei geraak word, nl. epilepsie pasiënte, pasiënte wat kalsiumblokkeerders ontvang agv 'n siektetoestand en orgaan oorplant pasiënte wat siklosporien behandeling ontvang vir onderdrukking van die weefselverwerpingsreaksie. Van hierdie genoemde pasiënte is slegs sowat 20 - 50 % vatbaar vir geneesmiddel geïnduseerde oorgroei van tandvleisweefsel. Die rede vir hierdie groep se vatbaarheid is nie duidelik. Hierdie navorsing is daarop gemik om die weefselreaksies op geneesmiddel toediening en die meganismes wat oorgroei veroorsaak beter te verstaan om uiteindelik vatbare pasiënte vooraf te kan identifiseer en voortydig die nodige maatreëls in te stel om die ongerief en komplikasies agv oorgroei wat by hierdie pasiënte waargeneem word uit te skakel of tot die minimum te beperk.

Omdat u gedurende die voorafgaande ondersoek geïdentifiseer is as 'n pasiënt wat behoort aan een van die genoemde groepe het ons u hulp en toestemming nodig om ons projek te laat slaag. Ten einde ons instaat te stel om al die variasies te kan bestudeer en ook kontrole maatreëls in te bou, is dit nodig dat ons weefselbiopsies (stukkies weefsel) vanaf 'n aantal pasiënte moet versamel. Hierdie prosedure sal behels dat die tandarts aan diens 'n klein stukkie tandvleisweefsel (1 mm^2) sal verwyder wat ons instaat sal stel om dit vir verdere navorsing te gebruik. Die letsel wat deur hierdie prosedure gelaat word sal binne enkele dae genees. Omdat hierdie prosedure 'n kliniese ingreep behels versoek ons u hiermee om toestemming te gee dat die tandarts aan diens 'n stukkie weefsel uit u mondholte kan verwyder. Die weefsel sal in verdere navorsing sonder bekendmaking van u identiteit of enige assosiasie met u spesifieke persoon gebruik word.

U vriendelike samewerking sal waardeer word.

Met vriendelike groete

Dr S.J. Botha
Senior Navorsingsbeampte en Projekleier
Sentrum vir Stomatologiese Navorsing

Prof W.J.C. Coetzee
Hoof en Voorsitter Etiese Komitee
Sentrum vir Stomatologiese Navorsing

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Faculty of Dentistry

1 March 1996

Dear Patient

RESEARCH PROJECT REGARDING HYPERPLASIA OF GINGIVAL TISSUE
CONSENT FOR TISSUE BIOPSY TO BE TAKEN

The Centre for Stomatological Research is undertaking a study of the effects of certain medications with regard to the overgrowth of the gingiva (gums). There are three main groups of patients affected by this condition namely: patients with epilepsy, patients taking calcium blockers and organ transplant patients taking cyclosporin. Of all these patients only about 20 - 50 % have this condition. The reason for this overgrowth is not clear. We are attempting to determine the reactions of the gingiva to the different medications and the mechanisms that cause their overgrowth. It is hoped to find a way of identifying those people who have the potential to develop this condition of the gingiva and to take the neccessary precautions to limit or prevent this overgrowth.

You have been identified as a patient falling within one of the main groups mentioned and your assistance in the study would be appreciated. To enable us to examine the factors involved in gingival overgrowth we need a small sample of gingival tissue. This involves the removal of a very small piece of gingiva which will heal up in a few days. The removal of this gingiva entails a surgical procedure and thus require your consent. The gingival tissue will be studied without any mention of your identity.

Your friendly co-operation would be appreciated.

Yours sincerely

Dr SJ Botha
Senior Research Officer and Project leader
Centre for Stomatological Research

Prof WJC Coetzee
Head and Chairman Ethics Committee
Centre for Stomatological Research

ICN Biomedicals, Inc.
1263 S. Chillicothe Rd.
Aurora, Ohio 44202
Telephone: 216/562-1500
FAX: 216/562-2642

Chemical Credential

Anti-Human Collagen Type IV Mouse Monoclonal Antibody Purified

Description: This antibody is supplied in 0.1 M Sodium Phosphate Buffer, pH 7.0. It is purified from ascites fluid in a concentration of 1 mg/ml. Each lot is characterized by SDS-PAGE and Western Blotting.

Clone/Isotype: Clone IV-4H12. Isotype mouse $IgG_{1/k}$.

Immunogen: Type IV Collagen from human placental tissue.

Reactivity/ Specificity: This antibody specifically reacts with Human Type IV Collagen.

Applications: Suitable for immunohistological procedures and Western Blotting.

References:

1. Mayne, R. et al., Isolation and Partial Characterization of Basement Membrane-link Collagens from Bovine Thoracic Aorta, **Artery** 7, 262-280, 1980.

2. Matsumoto, E. et al., Foreknowledge of Liver Fibrosis: Development of Immunoassay System for Serum Collagen Peptides with Monoclonal Antibody, J. Wakayaka, **Med. Soc.** 39, 87-106, 1988, (in Japanese).

Storage: Aliquot into convenient working portions and store undiluted at -20°C. Avoid freeze/thaw cycles as this is detrimental to most proteins.

Manufactured for ICN Biomedicals by Fuji Chemical Industries, Ltd.

PRODUCT: Anti-Human Collagen Type IV Monoclonal

Approved: Dr. Carl Grove

CAT. NO: 63-173

LOT NO: 80151

CONTROL: R077

ICN Biomedicals, Inc.
1263 S. Chillicothe Rd.
Aurora, Ohio 44202
Telephone: 216/562-1500
FAX: 216/562-2642

Chemical Credential

Anti-Human Collagen Type V Mouse Monoclonal Antibody Purified

Description: This antibody is supplied in 0.1 M Sodium Phosphate Buffer, pH 7.0. It is purified from ascites fluid in a concentration of 1 mg/ml. Each lot is characterized by SDS-PAGE and Western Blotting.

Clone/Type: Clone V-3C9. Type mouse IgM_k.

Immunogen: Type V Collagen from human placental tissue.

Reactivity/ Specificity: This antibody specifically reacts with Human Type V Collagen. It also cross-reacts with rabbit and rat Type V Collagen.

Applications: Suitable for immunohistological procedures and Western Blotting.

References:

1. Mayne, R. et al., Isolation and Partial Characterization of Basement Membrane-link Collagens from Bovine Thoracic Aorta, **Artery 7**, 262-280, 1980.

2. Niyibizi, C. et al., Human Placenta Type V Collagens. Evidence for the Existence of an α1(V), α2(V) and α3(V) Collagen Molecule. **J. Biol. Chem. 259**, 14170-14174, 1984.

Storage: Aliquot into convenient working portions and store undiluted at -20°C. Avoid freeze/thaw cycles as this is detrimental to most proteins.

Manufactured for ICN Biomedicals by Fuji Chemical Industries, Ltd.

PRODUCT: Anti-Human Collagen Type V Monoclonal

Approved: Dr. Carol Brauer

CAT. NO: 63-174

LOT NO: 80152

CONTROL: R077

ICN Biomedicals, Inc.
1263 S. Chillicothe Rd.
Aurora, Ohio 44202
Telephone: 216/562-1500
FAX: 216/562-2642

Chemical Credential

Anti-Human Collagen Type VI Mouse Monoclonal Antibody Purified

Description: This antibody is supplied in 0.1 M Sodium Phosphate Buffer, pH 7.0. It is purified from ascites fluid in a concentration of 1 mg/ml. Each lot is characterized by SDS-PAGE and Western Blotting.

Clone/Isotype: Clone VI-26. Isotype mouse $IgG_{1/k}$.

Immunogen: Type VI Collagen from human placental tissue.

Reactivity/ Specificity: This antibody specifically reacts with Human Type VI Collagen. It also recognizes rabbit and rat Type VI Collagen.

Applications: Suitable for immunohistological procedures and Western Blotting.

References:

1. Mayne, R. et al., Isolation and partial characterization of basement membrane-link collagens from bovine thoracic aorta, **Artery** 7, 262-280, 1980.

2. Muragaki, Y. et al., Experimental study on alteration of collagen metabolism in liver fibrosis, J. Wakayama **Med. Soc.** 36, 195-208, 1985, (in Japanese).

3. Hessle, H. and Engvall, E. Type VI Collagen, Studies on its localization, structure and biosynthetic form with monoclonal antibodies, **J. Biol. Chem.** 259, 3955-3961, 1984.

Storage: Aliquot into convenient working portions and store undiluted at -20°C. Avoid freeze/thaw cycles as this is detrimental to most protein.

Manufactured for ICN Biomedicals by Fuji Chemical Industries, Ltd.

PRODUCT: Anti-Human Collagen Type VI Monoclonal

Approved: Dr Carl Crowe

CAT. NO: 63-175

LOT NO: 80153

CONTROL: R077

ICN Biomedicals, Inc.
1263 S. Chillicothe Rd.
Aurora, Ohio 44202
Telephone: 216/562-1500
FAX: 216/562-2642

Chemical Credential

Anti-Human Collagen Type III Mouse Monoclonal Antibody Purified

Description: This antibody is supplied in 0.1 M Sodium Phosphate Buffer, pH 7.0. It is purified from ascites fluid in a concentration of 1 mg/ml. Each lot is characterized by SDS-PAGE and Western Blotting.

Clone/Isotype: Clone III-53. Isotype mouse $IgG_{1/k}$.

Immunogen: Type III Collagen from human placental tissue.

Reactivity/ Specificity: This antibody specifically reacts with Human Type III Collagen. It also cross-reacts with rabbit.

Applications: Suitable for immunohistological procedures and Western Blotting.

Storage: Aliquot into convenient working portions and store undiluted at -20°C. Avoid freeze/thaw cycles as this is detrimental to most proteins.

Manufactured for ICN Biomedicals by Fuji Chemical Industries, Ltd.

PRODUCT: Anti-Human Collagen Type II Monoclonal

Approved: Dr. Carol Olsen

CAT. NO: 63-172

LOT NO: 80150

CONTROL: R077

Conditions:
The product described here should not be administered to humans or used for any drug purpose. This product is without warranty other than information specifically provided in this Chemical Credential. No other warranty is made, expressed or implied, including but not limited to the warranties of merchantability and fitness for a particular purpose.

Addendum C: Cell counts

Tabel C.1.

Results obtained after preparation of single cell suspensions and determination of cell concentrations of the different series of wells over a period of ten weeks.

Group: Control = 1; Responder = 2; Non-responder = 3

Patient: Control 1-5; Responder 1-5; Non-responder 1-5

Treatment: 1-3 where 1 = MEM; 2 = CYC; 3 = CA

Loc: 1-20

				t0	t1	t2	t3	t4	t6	t6	t7	t8	t9	t10	t11	t12	t13	t14	t15	t16	t17	t18	t19	t20
Group	Patient	Treat	Loc	24H	96H	168H	264H	336H	432H	504H	576H	648H	744H	816H	912H	984H	1080H	1152H	1248H	1320H	1416H	1488H	1584H	1656H
1	1	1	1	1	3	1	1	2	1	5	4	6	5	9	7	8	5	5	13	11	5	4	7	8
1	1	1	2	3	2	6	2	1	4	4	3	6	4	6	5	9	5	3	7	8	5	5	13	5
1	1	1	3	1	3	1	1	0	3	7	3	6	5	6	6	14	4	4	13	8	9	4	13	5
1	1	1	4	0	3	2	2	0	6	5	4	4	7	4	9	10	5	3	7	8	6	5	4	8
1	1	1	5	2	3	1	4	3	3	6	8	3	3	9	7	13	4	6	12	7	9	3	8	8
1	1	1	6	3	4	2	2	0	3	6	3	6	5	5	8	6	7	6	7	8	10	5	5	6
1	1	1	7	1	3	3	2	3	3	5	6	6	5	4	7	7	5	4	7	5	4	3	12	7
1	1	1	8	0	1	3	2	0	5	4	6	3	4	4	7	9	3	10	9	5	5	4	9	8
1	1	1	9	0	4	3	1	4	3	4	7	3	3	6	6	7	5	6	11	11	5	5	7	6
1	1	1	10	2	4	3	2	1	2	4	5	5	5	6	5	8	3	3	9	7	4	5	15	9
1	1	1	11	5	2	1	5	0	1	5	9	9	5	5	9	8	13	3	9	6	4	5	6	11
1	1	1	12	2	3	2	2	3	1	3	8	6	5	3	13	8	5	6	11	8	4	7	6	8
1	1	1	13	3	1	2	1	0	6	7	5	9	5	5	8	6	4	6	8	7	4	5	8	6
1	1	1	14	3	2	5	1	0	2	4	5	4	5	6	7	5	7	4	7	8	3	6	6	12
1	1	1	15	1	2	2	3	2	3	5	3	8	5	5	6	6	3	5	10	12	5	4	8	8
1	1	1	16	4	2	3	5	2	1	5	11	7	5	3	7	8	5	3	9	6	9	4	10	8
1	1	1	17	1	2	3	1	0	3	8	4	8	7	7	9	5	3	3	7	11	6	4	8	11
1	1	1	18	4	2	3	2	0	6	4	5	5	7	5	4	6	3	5	8	9	3	8	8	11
1	1	1	19	2	2	4	2	0	3	4	4	7	7	5	4	11	5	5	7	8	3	5	8	6
1	1	1	20	4	1	1	2	8	1	6	4	8	5	3	5	6	7	5	9	6	3	11	5	5
			Ave	2.10	2.47	2.63	2.19	1.45	3.00	5.06	5.39	5.94	5.13	5.38	6.94	8.00	5.07	4.71	9.00	7.93	5.33	5.13	8.40	7.76

Group	Patient	Treat	Loc	t0 24H	t1 96H	t2 168H	t3 264H	t4 336H	t5 432H	t6 504H	t7 576H	t8 648H	t9 744H	t10 816H	t11 912H	t12 984H	t13 1080H	t14 1152H	t15 1248H	t16 1320H	t17 1416H	t18 1488H	t19 1584H	t20 1656H
1	2	1	1	1	2	6	3	5	4	7	16	11	12	10	10	26	9	9	18	10	18	14	16	7
1	2	1	2	1	1	6	7	5	5	7	11	7	8	14	12	16	9	6	14	12	13	17	9	13
1	2	1	3	0	1	6	3	6	3	7	11	13	10	9	12	23	9	9	14	14	16	11	10	9
1	2	1	4	1	2	9	9	5	6	9	12	13	6	6	12	26	8	9	13	14	16	13	13	10
1	2	1	5	1	2	7	8	3	5	7	11	10	7	11	10	29	9	9	10	14	13	13	13	14
1	2	1	6	0	5	6	8	3	3	7	14	12	10	7	9	21	10	7	13	14	16	12	16	8
1	2	1	7	0	4	7	3	5	4	6	8	8	8	12	10	19	9	7	10	11	18	10	13	7
1	2	1	8	2	1	6	6	5	5	6	12	22	11	8	12	27	8	9	12	10	17	13	12	6
1	2	1	9	0	2	6	3	4	6	7	12	7	10	6	10	22	9	9	9	12	15	15	11	5
1	2	1	10	1	3	7	5	3	7	9	8	13	12	10	10	32	8	12	20	14	13	12	11	15
1	2	1	11	0	3	10	7	4	3	5	7	11	10	8	12	15	12	8	15	21	20	13	13	6
1	2	1	12	1	1	10	5	5	5	10	10	10	10	14	15	19	10	6	15	19	16	12	17	7
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1	2	1	15	0	4	12	3	5	10	5	11	15	10	9	12	17	7	9	12	18	14	14	11	6
1	2	1	16	2	3	8	5	12	5	6	15	8	9	10	17	22	11	12	13	15	18	11	16	8
1	2	1	17	1	1	6	5	5	5	5	11	8	7	7	17	22	12	9	9	16	17	14	13	6
1	2	1	18	1	3	6	4	6	2	5	11	11	8	9	15	16	9	14	11	17	18	10	14	5
1	2	1	19	4	1	6	5	5	6	7	11	11	10	9	9	22	8	12	13	14	13	13	13	6
1	2	1	20	1	3	9	4	9	4	7	8	10	11	9	12	22	8	13	13	14	16	12	12	5
			Ave	0.95	2.48	7.29	5.18	5.23	4.90	6.68	10.93	11.08	9.41	9.23	11.80	22.00	9.26	9.43	12.74	14.13	15.91	12.98	12.86	7.88

Group	Patient	Treat	Loc	t0 24H	t1 96H	t2 168H	t3 264H	t4 336H	t5 432H	t6 504H	t7 576H	t8 648H	t9 744H	t10 816H	t11 912H	t12 984H	t13 1080H	t14 1152H	t15 1248H	t16 1320H	t17 1416H	t18 1488H	t19 1584H	t20 1656H
1	2	2	1	2	2	6	6	6	5	8	7	14	5	9	6	9	8	10	10	7	4	8	7	6
1	2	2	2	2	2	6	8	5	8	4	4	7	6	11	4	6	5	11	10	5	9	6	14	5
1	2	2	3	4	1	1	6	3	11	4	4	10	6	10	7	8	6	8	10	9	5	5	8	6
1	2	2	4	2	1	3	3	3	8	9	5	8	4	5	8	8	8	11	8	7	4	10	8	12
1	2	2	5	2	3	7	6	4	7	6	4	10	4	9	4	10	7	9	9	6	7	6	9	8
1	2	2	6	4	2	3	6	4	7	6	3	13	7	8	9	8	7	11	10	7	7	6	14	7
1	2	2	7	1	2	2	6	6	7	4	5	12	6	9	6	10	4	12	17	9	8	7	9	5
1	2	2	8	3	1	3	5	5	8	3	4	14	4	9	6	8	6	10	12	6	7	5	9	8
1	2	2	9	4	1	3	4	5	8	9	5	10	8	10	7	8	7	11	10	8	7	6	8	8
1	2	2	10	3	1	3	5	5	11	3	4	11	4	7	7	9	7	8	12	8	5	6	7	10
1	2	2	11	4	2	3	8	6	11	5	3	7	5	7	12	9	7	9	13	8	5	4	8	7
1	2	2	12	1	4	2	8	5	8	9	5	9	10	9	8	7	7	7	8	5	4	5	9	8
1	2	2	13	2	2	3	6	4	7	7	6	7	10	11	5	8	5	11	11	7	5	8	8	6
1	2	2	14	2	3	4	3	7	7	5	7	11	8	11	4	7	9	19	8	7	7	6	8	8
1	2	2	15	1	3	1	8	4	8	4	5	11	6	5	4	9	7	11	9	8	7	6	8	13
1	2	2	16	3	4	2	7	7	9	5	5	10	6	13	6	8	6	12	10	9	8	9	10	5
1	2	2	17	2	3	5	6	9	9	3	3	8	6	9	5	9	11	13	12	8	9	4	9	8
1	2	2	18	4	3	4	7	4	5	4	3	10	5	9	6	8	4	9	10	5	10	6	14	10
1	2	2	19	2	3	3	5	5	11	3	7	9	9	10	6	8	8	13	7	5	8	5	9	11
1	2	2	20	0	2	3	5	5	8	5	6	10	9	9	7	7	7	8	10	7	10	4	9	7
			Ave	2.40	2.28	3.41	5.87	5.12	8.20	5.33	4.89	10.06	6.47	9.00	6.47	6.27	6.73	10.59	10.43	7.06	6.76	6.13	9.26	7.87

Group	Patient	Treat	Loc	t0 24H	t1 96H	t2 188H	t3 264H	t4 336H	t5 432H	t6 504H	t7 576H	t8 648H	t9 744H	t10 816H	t11 912H	t12 984H	t13 1080H	t14 1152H	t15 1248H	t16 1320H	t17 1416H	t18 1488H	t19 1584H	t20 1656H
1	1	2	1	6	4	3	4	3	5	6	13	5	7	8	9	6	4	9	12	15	9	8	7	10
1	1	2	2	1	6	2	5	2	4	5	7	8	7	7	9	7	7	9	10	13	4	7	8	7
1	1	2	3	4	5	4	4	1	5	5	5	8	8	7	11	13	4	8	9	10	6	6	8	12
1	1	2	4	4	5	3	4	5	3	6	6	7	6	7	9	11	7	7	12	17	6	4	8	10
1	1	2	5	4	6	4	4	2	5	4	6	6	6	7	9	9	7	8	11	16	6	9	8	10
1	1	2	6	3	4	6	9	3	5	6	10	6	6	10	9	11	6	11	11	21	6	4	8	8
1	1	2	7	2	6	2	6	4	3	4	9	5	7	13	7	9	7	6	12	13	4	4	8	10
1	1	2	8	3	5	4	5	3	9	8	5	5	6	9	9	8	10	7	7	12	6	4	8	8
1	1	2	9	4	3	4	4	2	4	4	7	5	9	4	9	7	11	9	6	12	6	6	9	7
1	1	2	10	3	7	4	4	6	5	3	5	6	13	6	8	8	7	12	10	8	6	8	8	9
1	1	2	11	4	6	6	5	5	4	5	7	6	9	7	17	7	8	6	9	12	8	7	9	8
1	1	2	12	0	4	4	3	1	5	3	7	3	6	10	11	9	7	7	17	7	7	6	6	9
1	1	2	13	0	5	5	6	4	5	6	7	8	6	9	8	8	4	9	9	12	4	4	8	7
1	1	2	14	5	5	3	3	3	5	5	7	4	6	6	7	9	6	6	10	12	6	6	9	13
1	1	2	15	1	8	4	5	5	4	5	7	5	8	6	8	9	9	6	9	9	11	4	8	13
1	1	2	16	0	9	2	4	1	8	5	6	3	9	6	7	13	4	6	7	10	7	11	12	10
1	1	2	17	3	5	6	3	1	6	8	7	3	7	7	10	6	6	6	12	12	6	7	7	8
1	1	2	18	3	3	5	3	1	9	4	6	6	7	4	9	8	7	6	10	7	6	6	7	17
1	1	2	19	4	3	2	3	2	4	3	11	3	7	7	8	8	6	7	10	6	6	4	6	17
1	1	2	20	4	5	9	3	2	4	5	5	4	6	6	6	8	7	6	10	8	9	7	11	8
			Ave	2.90	5.25	4.11	4.41	2.79	5.12	5.00	7.18	5.40	7.14	7.28	8.89	8.51	6.56	7.49	10.22	11.53	6.50	6.23	8.08	10.01

Group	Patient	Treat	Loc	t0 24H	t1 96H	t2 188H	t3 264H	t4 336H	t5 432H	t6 504H	t7 576H	t8 648H	t9 744H	t10 816H	t11 912H	t12 984H	t13 1080H	t14 1152H	t15 1248H	t16 1320H	t17 1416H	t18 1488H	t19 1584H	t20 1656H
1	1	3	1	6	6	4	4	6	3	4	9	4	9	4	12	9	4	6	12	11	7	3	5	7
1	1	3	2	3	3	3	7	4	5	9	9	6	5	5	13	8	4	4	11	7	7	5	5	13
1	1	3	3	1	3	6	8	6	2	9	12	5	7	9	5	10	8	5	6	9	8	3	5	9
1	1	3	4	0	7	4	5	3	2	4	8	4	4	7	5	9	3	5	7	9	6	5	5	10
1	1	3	5	0	7	10	7	4	2	4	7	6	6	9	11	12	7	3	7	8	10	5	4	14
1	1	3	6	6	4	4	10	15	4	5	8	6	6	9	7	12	3	5	12	10	5	3	5	8
1	1	3	7	4	7	3	6	9	4	6	5	8	6	10	6	9	9	5	8	9	10	6	8	7
1	1	3	8	1	4	8	8	8	3	5	8	4	4	8	7	6	7	5	7	6	7	6	5	6
1	1	3	9	5	6	5	5	7	3	9	8	4	6	8	6	6	11	3	7	10	5	5	6	5
1	1	3	10	0	5	5	5	11	4	7	7	6	4	8	7	9	6	4	9	9	5	10	9	15
1	1	3	11	1	3	7	4	6	3	6	6	6	4	10	9	11	6	8	7	9	7	5	5	6
1	1	3	12	3	5	5	4	4	2	6	11	6	6	8	6	6	8	9	11	10	5	5	5	7
1	1	3	13	3	4	3	6	3	4	4	7	5	6	9	7	13	5	3	6	10	7	6	6	7
1	1	3	14	3	5	5	4	3	6	6	8	6	5	8	6	9	5	4	6	9	6	11	4	8
1	1	3	15	3	4	7	6	6	6	8	8	6	4	6	5	8	6	7	8	11	11	3	4	6
1	1	3	16	0	3	6	4	5	3	6	8	14	6	12	7	11	3	5	12	6	6	3	5	8
1	1	3	17	2	4	4	7	6	3	8	9	6	5	6	6	10	6	3	12	10	5	6	6	6
1	1	3	18	3	5	5	7	3	5	7	7	10	9	5	8	11	3	6	6	9	5	7	5	5
1	1	3	19	3	3	4	7	3	8	7	7	6	6	6	7	9	8	3	6	8	9	4	4	6
1	1	3	20	5	5	3	5	7	4	6	10	5	4	7	7	6	6	6	6	6	9	4	6	5
			Ave	2.60	4.61	5.06	5.94	5.94	3.78	6.33	8.13	8.18	5.50	7.60	7.39	9.29	5.89	4.94	8.33	8.73	7.00	5.29	5.44	7.89

Group	Patient	Treat	Loc	t0 24H	t1 96H	t2 168H	t3 264H	t4 336H	t5 432H	t6 504H	t7 576H	t8 648H	t9 744H	t10 816H	t11 912H	t12 984H	t13 1080H	t14 1152H	t15 1248H	t16 1320H	t17 1416H	t18 1488H	t19 1584H	t20 1656H
1	2	3	1	1	3	7	6	7	14	5	11	14	8	13	14	12	13	7	14	17	10	6	13	16
1	2	3	2	3	3	9	4	7	8	5	14	7	10	13	14	19	8	9	14	13	9	7	10	15
1	2	3	3	4	5	7	8	6	8	8	10	10	11	18	14	22	9	12	15	10	9	7	13	10
1	2	3	4	1	3	10	8	6	6	10	10	8	11	12	14	13	12	9	13	13	10	7	11	13
1	2	3	5	3	2	9	3	4	12	8	7	10	11	15	14	16	12	9	13	9	11	7	13	18
1	2	3	6	1	2	9	6	3	6	8	10	13	11	16	13	15	9	6	18	9	9	8	11	14
1	2	3	7	3	4	7	6	6	9	8	10	12	11	15	12	16	9	8	11	15	9	7	12	13
1	2	3	8	4	2	9	4	6	6	5	7	14	10	10	14	14	12	11	14	13	17	6	8	13
1	2	3	9	2	2	7	6	9	6	5	7	10	10	13	12	13	13	6	14	13	10	8	14	13
1	2	3	10	4	5	9	6	4	9	8	11	11	11	12	14	12	10	8	12	15	11	7	11	13
1	2	3	11	1	6	14	9	8	11	14	8	7	8	13	19	13	8	7	13	15	14	14	11	11
1	2	3	12	4	4	12	6	5	13	15	7	9	12	16	12	16	16	10	17	14	11	8	10	13
1	2	3	13	3	3	13	7	6	11	6	12	7	11	10	19	17	11	10	13	15	11	5	13	13
1	2	3	14	3	2	10	5	6	9	8	7	11	12	11	10	15	16	11	14	10	11	7	9	13
1	2	3	15	1	2	13	4	6	10	5	19	11	11	13	23	15	12	9	10	9	13	8	11	10
1	2	3	16	2	2	7	5	4	11	9	18	10	17	9	15	11	8	13	12	12	10	10	11	14
1	2	3	17	5	3	9	5	3	6	8	12	8	12	13	11	22	13	6	14	17	11	8	11	11
1	2	3	18	6	4	10	7	4	6	11	8	10	10	11	12	15	17	9	12	10	15	11	11	11
1	2	3	19	4	4	9	6	7	11	5	8	9	11	13	12	15	13	11	15	10	11	13	13	15
1	2	3	20	3	3	7	5	6	9	10	10	10	7	13	12	15	14	12	15	13	10	5	8	12
			Ave	2.90	3.25	9.47	5.76	5.59	9.06	8.07	10.38	10.06	10.67	12.93	14.00	15.40	11.71	9.19	13.56	12.50	11.12	7.94	11.27	13.07

Group	Patient	Treat	Loc	t0 24H	t1 96H	t2 168H	t3 264H	t4 336H	t5 432H	t6 504H	t7 576H	t8 648H	t9 744H	t10 816H	t11 912H	t12 984H	t13 1080H	t14 1152H	t15 1248H	t16 1320H	t17 1416H	t18 1488H	t19 1584H	t20 1656H
1	3	1	1	1	3	6	16	3	4	14	14	18	32	18	17	29	18	19	25	41	19	25	18	28
1	3	1	2	2	4	6	14	11	8	13	11	22	31	18	12	21	21	17	29	35	16	29	21	28
1	3	1	3	2	4	6	14	3	7	10	13	22	24	26	13	19	18	19	27	26	21	21	18	28
1	3	1	4	2	5	4	12	6	8	12	14	22	21	19	13	21	18	19	27	35	20	23	18	27
1	3	1	5	4	5	5	13	3	4	17	13	22	20	21	15	20	23	19	27	29	21	17	16	28
1	3	1	6	0	5	13	14	7	5	18	15	22	24	21	12	18	12	24	29	25	19	22	17	28
1	3	1	7	3	3	4	14	6	6	10	16	15	20	21	15	17	22	18	26	25	17	21	18	31
1	3	1	8	3	6	4	14	5	8	13	19	17	22	26	12	21	22	22	28	25	19	25	17	28
1	3	1	9	2	3	5	13	5	4	10	12	16	23	25	14	16	21	17	23	31	19	21	20	28
1	3	1	10	2	4	6	14	3	4	12	17	22	19	26	16	18	17	14	27	27	22	20	22	25
1	3	1	11	1	11	5	10	10	7	10	14	29	20	21	13	19	19	21	26	34	19	17	22	28
1	3	1	12	7	5	7	13	7	6	15	18	28	29	21	18	23	26	18	31	26	19	21	16	32
1	3	1	13	1	6	6	10	3	6	13	14	22	18	21	17	21	23	19	27	37	24	17	21	23
1	3	1	14	1	8	6	15	6	10	20	14	20	22	19	12	19	21	22	23	25	17	24	16	30
1	3	1	15	3	5	7	11	6	6	11	10	18	23	21	14	20	22	15	25	29	18	21	19	32
1	3	1	16	3	5	6	13	9	6	13	14	28	23	16	15	22	30	16	33	29	19	17	15	23
1	3	1	17	2	4	6	13	5	8	14	11	20	23	20	15	24	17	17	27	29	20	20	18	22
1	3	1	18	1	5	4	12	4	4	13	10	27	23	18	20	21	21	19	29	29	16	19	17	21
1	3	1	19	1	4	6	11	8	6	13	17	26	23	20	19	23	21	21	27	25	16	24	18	32
1	3	1	20	2	3	6	10	4	6	13	8	20	23	21	16	30	25	21	27	25	22	18	16	34
			Ave	2.15	4.87	5.88	12.76	5.65	6.20	13.27	13.57	21.71	23.21	20.93	14.88	21.13	20.82	18.79	27.23	29.47	19.19	21.13	18.21	27.67

Group	Patient	Treat	Loc	t0 24H	t1 96H	t2 168H	t3 264H	t4 336H	t5 432H	t6 504H	t7 576H	t8 648H	t9 744H	t10 816H	t11 912H	t12 984H	t13 1080H	t14 1152H	t15 1248H	t16 1320H	t17 1416H	t18 1488H	t19 1584H	t20 1656H
1	3	2	1	2	10	9	13	8	14	24	26	30	32	41	41	55	21	22	40	38	34	28	30	21
1	3	2	2	2	5	9	13	7	15	32	35	30	27	34	52	58	26	26	36	38	37	50	42	23
1	3	2	3	1	6	11	13	12	13	23	30	32	31	29	40	40	35	25	38	43	45	33	45	23
1	3	2	4	5	12	9	8	13	15	18	26	35	26	34	47	45	25	21	39	38	38	39	40	27
1	3	2	5	1	9	9	13	12	18	24	26	50	28	29	48	42	19	21	39	36	36	29	34	23
1	3	2	6	2	10	8	10	12	12	19	26	30	31	36	48	38	26	26	50	35	37	32	29	29
1	3	2	7	4	5	8	13	10	12	21	21	26	32	30	45	40	26	26	35	35	44	33	46	25
1	3	2	8	2	10	9	12	8	12	24	29	30	32	27	48	42	22	23	39	35	32	35	53	23
1	3	2	9	1	6	9	13	9	14	23	23	30	32	33	48	50	21	26	32	35	36	27	33	32
1	3	2	10	0	6	6	15	13	14	20	25	30	24	33	40	43	23	25	39	35	37	33	36	23
1	3	2	11	10	4	12	12	15	14	22	28	46	32	36	52	45	26	32	39	27	31	34	29	20
1	3	2	12	7	4	7	13	12	14	24	26	29	39	39	61	61	28	29	48	42	37	33	34	18
1	3	2	13	3	7	7	10	13	17	25	23	20	42	33	43	47	27	29	49	38	47	33	36	23
1	3	2	14	5	6	6	12	7	14	23	23	28	32	29	45	38	26	26	39	37	40	30	32	21
1	3	2	15	3	7	9	14	15	14	23	25	24	24	35	48	45	29	21	40	29	37	27	36	18
1	3	2	16	4	4	11	16	18	17	23	26	30	43	33	67	38	35	32	32	35	37	36	33	23
1	3	2	17	1	4	10	9	12	11	20	22	24	38	33	43	45	21	31	32	30	43	35	36	23
1	3	2	18	2	5	13	9	15	13	22	28	24	40	33	51	45	36	26	40	34	34	33	28	19
1	3	2	19	3	6	9	26	9	20	26	26	30	27	31	43	45	22	25	38	35	33	35	34	26
1	3	2	20	5	7	6	23	15	14	23	26	21	32	30	48	40	23	27	38	29	31	31	36	23
			Ave	3.15	6.61	8.80	13.47	11.67	14.47	22.93	26.00	29.93	32.29	32.87	47.86	45.13	25.80	25.93	39.13	35.27	37.40	33.40	36.13	23.21

Group	Patient	Treat	Loc	t0 24H	t1 96H	t2 168H	t3 264H	t4 336H	t5 432H	t6 504H	t7 576H	t8 648H	t9 744H	t10 816H	t11 912H	t12 984H	t13 1080H	t14 1152H	t15 1248H	t16 1320H	t17 1416H	t18 1488H	t19 1584H	t20 1656H
1	3	3	1	1	6	4	16	7	16	15	12	25	34	22	23	32	23	21	39	23	24	32	32	26
1	3	3	2	2	7	7	14	9	17	15	12	17	27	25	23	28	28	21	49	25	21	28	32	26
1	3	3	3	1	5	6	16	17	14	12	15	20	30	24	18	32	26	22	50	22	21	24	26	26
1	3	3	4	2	4	7	16	13	22	11	10	20	21	26	23	37	26	22	49	28	20	23	25	27
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1	3	3	19	4	4	7	24	8	11	25	15	20	18	27	31	29	29	21	41	24	24	26	35	27
1	3	3	20	1	4	9	17	11	9	15	16	20	26	27	22	30	27	22	58	26	21	21	32	30
			Ave	1.50	5.00	7.08	15.57	10.88	12.94	14.93	15.00	20.13	26.07	26.53	22.67	32.21	26.86	22.00	48.69	26.43	24.00	24.89	32.36	27.33

Group	Patient	Treat	Loc	t0 24H	t1 96H	t2 168H	t3 264H	t4 336H	t5 432H	t6 504H	t7 576H	t8 648H	t9 744H	t10 816H	t11 912H	t12 984H	t13 1080H	t14 1152H	t15 1248H	t16 1320H	t17 1416H	t18 1488H	t19 1584H	t20 1656H
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1	4	1	2	1	5	4	5	1	5	9	9	6	16	15	16	10	38	16	17	13	16	25	19	22
1	4	1	3	1	3	4	6	4	8	8	9	11	19	14	16	11	22	11	12	12	22	20	19	18
1	4	1	4	2	2	5	3	3	4	12	6	9	21	13	16	12	24	9	15	16	18	22	16	18
1	4	1	5	0	6	6	9	4	3	14	8	8	19	9	21	16	20	14	18	13	17	20	19	19
1	4	1	6	2	6	4	8	4	5	12	12	11	24	12	16	16	22	12	18	15	15	17	13	20
1	4	1	7	0	2	4	8	2	4	14	5	12	20	15	11	13	22	12	14	13	16	28	14	16
1	4	1	8	1	3	4	3	6	3	12	14	16	22	10	18	13	21	10	18	10	16	17	11	21
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1	4	1	10	2	5	6	8	3	5	7	10	10	20	12	16	14	22	12	17	11	18	34	20	16
1	4	1	11	0	3	5	4	4	3	8	9	8	18	10	13	13	18	17	20	11	18	20	14	16
1	4	1	12	3	2	4	4	2	2	7	6	9	14	13	13	13	22	14	27	13	18	20	18	18
1	4	1	13	0	2	7	5	4	4	10	9	10	13	11	19	10	21	11	14	15	15	22	19	18
1	4	1	14	3	2	3	4	8	5	7	9	10	18	9	16	11	15	12	20	13	18	23	20	18
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1	4	1	17	1	3	3	5	3	5	10	9	10	13	12	13	12	31	10	18	15	18	23	23	21
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1	4	1	20	0	1	4	3	4	3	10	9	10	14	12	16	14	22	13	18	16	19	19	16	15
			Ave	1.25	2.94	4.24	5.33	3.75	4.06	9.64	8.67	10.17	16.00	11.87	16.07	12.60	22.00	12.29	18.07	13.13	18.47	22.41	18.94	18.33

Group	Patient	Treat	Loc	t0 24H	t1 96H	t2 168H	t3 264H	t4 336H	t5 432H	t6 504H	t7 576H	t8 648H	t9 744H	t10 816H	t11 912H	t12 984H	t13 1080H	t14 1152H	t15 1248H	t16 1320H	t17 1416H	t18 1488H	t19 1584H	t20 1656H
1	4	2	1	8	4	8	6	9	9	9	14	15	17	16	13	34	19	20	30	17	20	21	14	33
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1	4	2	3	1	3	10	8	4	7	7	20	23	16	13	13	21	20	15	31	24	18	16	24	38
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1	4	2	5	5	2	3	9	10	4	11	14	16	12	17	13	27	20	13	29	12	25	19	13	30
1	4	2	6	0	2	3	9	5	7	11	16	12	18	17	13	22	20	14	25	23	25	17	13	34
1	4	2	7	2	5	1	9	3	6	11	17	20	13	12	12	32	21	16	32	18	25	23	13	38
1	4	2	8	2	2	1	9	6	7	11	17	12	12	13	10	23	19	18	30	15	25	18	17	36
1	4	2	9	2	1	4	6	6	8	10	14	16	16	16	12	25	17	13	23	21	25	17	17	28
1	4	2	10	2	2	5	6	6	7	10	14	15	17	17	15	19	20	13	28	19	19	21	17	29
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1	4	2	12	2	4	2	10	4	7	9	9	17	19	17	12	20	21	13	24	17	23	23	19	32
1	4	2	13	3	2	3	12	7	6	8	13	14	20	17	15	25	16	13	24	17	30	19	17	32
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1	4	2	17	3	2	2	9	8	5	10	9	13	16	18	13	25	17	14	18	13	25	21	17	32
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1	4	2	20	2	4	6	6	3	5	15	12	13	16	19	19	32	30	14	13	17	22	16	19	19
			Ave	2.30	2.39	3.41	8.94	5.73	7.00	10.56	13.71	15.18	15.93	16.65	13.36	24.79	20.36	14.35	23.93	18.50	24.54	18.63	16.53	31.73

Group	Patient	Treat	Loc	t0 24H	t1 96H	t2 168H	t3 284H	t4 336H	t5 432H	t6 504H	t7 576H	t8 648H	t9 744H	t10 816H	t11 912H	t12 984H	t13 1080H	t14 1152H	t15 1248H	t16 1320H	t17 1416H	t18 1488H	t19 1584H	t20 1656H
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1	4	3	4	1	2	5	4	5	5	7	8	14	12	9	18	20	18	14	27	21	17	14	16	15
1	4	3	5	4	4	5	7	3	5	12	10	12	16	7	21	18	18	10	28	28	14	15	11	16
1	4	3	6	0	2	4	13	7	6	9	9	18	12	11	18	17	19	25	24	19	18	16	12	14
1	4	3	7	1	1	5	6	6	8	9	10	15	16	7	16	17	18	13	26	15	12	11	13	14
1	4	3	8	0	2	5	6	5	6	7	8	17	15	8	15	15	15	20	17	14	16	12	12	17
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1	4	3	13	3	1	5	6	5	8	7	7	19	21	11	15	16	17	14	19	18	13	14	15	17
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1	4	3	15	0	2	8	4	6	10	8	9	13	20	9	17	17	20	18	19	17	14	15	13	22
1	4	3	16	3	3	6	11	4	9	9	11	23	12	11	13	18	20	14	15	12	14	15	14	21
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1	4	3	18	2	5	5	4	5	8	9	8	19	18	10	15	14	18	11	13	17	14	12	14	15
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1	4	3	20	3	2	5	4	3	10	8	7	18	15	11	14	17	15	14	19	17	12	12	12	16
			Ave	2.00	2.65	4.60	6.00	5.18	7.53	9.35	8.94	17.73	16.06	9.24	15.36	17.27	20	14.40	18.92	18.67	14.05	14.71	13.19	16.56

Group	Patient	Treat	Loc	t0 24H	t1 96H	t2 168H	t3 284H	t4 336H	t5 432H	t6 504H	t7 576H	t8 648H	t9 744H	t10 816H	t11 912H	t12 984H	t13 1080H	t14 1152H	t15 1248H	t16 1320H	t17 1416H	t18 1488H	t19 1584H	t20 1656H
1	5	1	1	0	4	6	5	2	4	5	4	5	10	18	20	12	7	11	13	12	13	19	14	13
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1	5	1	4	1	4	5	7	5	3	8	11	10	10	7	21	12	11	8	13	11	11	17	13	13
1	5	1	5	0	6	6	5	5	6	8	9	5	7	7	15	8	8	10	17	18	9	10	16	13
1	5	1	6	1	4	7	5	8	5	9	7	5	10	10	14	14	10	7	15	8	11	13	26	13
1	5	1	7	0	4	15	5	4	4	8	7	7	8	7	21	10	13	11	13	13	11	10	17	18
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1	5	1	9	1	7	6	3	6	5	5	7	9	7	11	21	11	7	12	18	9	12	14	13	12
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1	5	1	15	1	4	5	5	10	5	8	4	6	12	11	19	12	9	10	10	16	14	16	17	12
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1	5	1	20	2	5	7	3	5	7	5	7	7	10	11	23	14	8	7	9	13	13	13	24	11
			Ave	0.75	4.47	7.06	4.81	5.00	4.81	6.78	7.24	6.79	10.40	10.18	21.31	11.87	9.11	9.71	12.87	12.06	11.00	13.07	16.27	12.93

Group	Patient	Treat	Loc	t0 24H	t1 96H	t2 168H	t3 284H	t4 338H	t5 432H	t6 504H	t7 576H	t8 648H	t9 744H	t10 816H	t11 912H	t12 984H	t13 1080H	t14 1152H	t15 1248H	t16 1320H	t17 1416H	t18 1488H	t19 1584H	t20 1656H
1	5	2	1	3	6	7	9	4	7	18	6	7	7	14	12	15	13	13	22	12	17	13	15	15
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1	5	2	3	2	8	7	12	2	10	5	6	7	21	13	12	14	11	14	13	12	16	13	16	11
1	5	2	4	0	5	6	12	5	9	5	9	11	14	13	16	15	14	14	23	15	17	13	16	8
1	5	2	5	0	6	9	13	6	8	12	9	6	10	13	15	20	14	12	24	9	14	12	12	10
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1	5	2	7	1	5	8	11	6	11	9	9	7	8	17	18	20	14	13	17	9	20	16	13	11
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1	5	2	9	2	4	6	12	3	10	8	5	5	10	13	10	20	13	13	19	14	19	12	14	11
1	5	2	10	5	7	7	12	7	9	9	8	7	12	12	15	20	14	13	19	12	22	13	14	11
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1	5	2	12	3	4	14	15	5	7	13	8	6	12	20	15	25	13	13	15	8	17	12	21	9
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1	5	2	20	6	7	6	17	6	10	9	5	8	8	10	28	24	19	13	13	12	13	12	15	15
			Ave	**2.35**	**5.73**	**8.39**	**12.40**	**5.12**	**9.53**	**9.40**	**7.88**	**7.29**	**11.13**	**12.73**	**14.93**	**19.53**	**14.13**	**13.27**	**16.69**	**11.50**	**16.73**	**13.06**	**16.06**	**10.93**

Group	Patient	Treat	Loc	t0 24H	t1 96H	t2 168H	t3 284H	t4 336H	t5 432H	t6 504H	t7 576H	t8 648H	t9 744H	t10 816H	t11 912H	t12 984H	t13 1080H	t14 1152H	t15 1248H	t16 1320H	t17 1416H	t18 1488H	t19 1584H	t20 1656H
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1	5	3	3	8	2	9	10	6	9	10	7	8	15	12	15	14	14	14	25	19	13	14	16	19
1	5	3	4	4	6	8	14	13	6	7	7	8	9	10	14	17	12	12	17	17	11	16	16	17
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1	5	3	9	7	4	6	10	8	9	6	10	8	11	6	14	17	14	15	17	20	14	11	17	15
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1	5	3	20	4	3	6	10	5	9	8	10	6	10	13	10	14	10	13	17	19	14	14	16	18
			Ave	**3.70**	**3.44**	**5.88**	**9.75**	**7.88**	**9.12**	**8.94**	**10.00**	**8.87**	**10.59**	**9.88**	**14.14**	**16.71**	**11.73**	**12.88**	**16.79**	**19.00**	**14.19**	**14.47**	**15.62**	**18.07**

Group	Patient	Treat	Loc	t0 24H	t1 96H	t2 168H	t3 264H	t4 336H	t5 432H	t6 504H	t7 576H	t8 648H	t9 744H	t10 816H	t11 912H	t12 984H	t13 1080H	t14 1152H	t15 1248H	t16 1320H	t17 1418H	t18 1488H	t19 1584H	t20 1656H
2	1	1	1	1	1	4	3	7	8	9	8	9	10	8	9	11	7	14	12	8	9	10	10	11
2	1	1	2	1	2	2	6	8	7	10	6	7	9	9	10	11	9	18	10	7	8	7	9	11
2	1	1	3	3	2	4	6	9	8	8	8	9	9	8	8	11	9	13	9	10	15	7	7	9
2	1	1	4	0	1	5	4	10	6	14	7	9	12	7	10	10	6	11	12	10	6	11	9	8
2	1	1	5	0	1	2	3	7	8	7	9	9	9	8	9	8	10	15	16	8	6	7	12	6
2	1	1	6	2	3	1	2	8	8	8	6	7	7	12	9	18	9	12	8	10	9	9	9	8
2	1	1	7	0	1	3	4	10	6	9	8	8	7	7	10	10	11	14	15	8	7	8	12	6
2	1	1	8	1	4	1	3	6	9	9	7	7	7	8	8	9	16	26	11	7	9	8	9	7
2	1	1	9	1	3	4	6	7	7	9	4	6	9	12	9	10	10	14	9	8	8	8	9	9
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2	1	1	11	1	1	3	9	8	8	12	4	6	9	10	13	11	11	10	12	8	8	7	8	10
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2	1	1	16	1	2	4	4	7	8	6	6	8	9	7	9	8	8	18	10	13	7	9	6	7
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2	1	1	18	0	2	3	4	8	8	11	4	6	8	7	10	15	9	17	17	9	11	7	6	8
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2	1	1	20	1	3	4	6	8	8	8	4	7	10	9	9	9	6	10	12	9	7	6	6	9
			Ave	0.80	1.94	2.72	4.38	6.13	6.19	9.20	6.44	7.70	8.76	6.36	9.71	10.99	9.03	14.14	12.32	8.40	7.84	7.63	8.82	7.84

Group	Patient	Treat	Loc	t0 24H	t1 96H	t2 168H	t3 264H	t4 336H	t5 432H	t6 504H	t7 576H	t8 648H	t9 744H	t10 816H	t11 912H	t12 984H	t13 1080H	t14 1152H	t15 1248H	t16 1320H	t17 1416H	t18 1488H	t19 1584H	t20 1656H
2	1	2	1	2	0	2	5	3	6	6	6	8	9	6	6	4	3	13	9	6	4	3	2	0
2	1	2	2	1	1	2	4	7	5	8	9	9	8	5	6	6	4	8	11	6	6	3	5	0
2	1	2	3	1	1	2	7	8	6	5	4	7	10	5	8	4	4	8	10	8	4	2	7	0
2	1	2	4	0	2	4	4	6	10	5	5	6	6	3	6	7	3	12	15	7	11	2	3	1
2	1	2	5	1	2	5	3	3	8	4	7	7	8	3	6	9	6	6	10	9	8	8	5	0
2	1	2	6	1	4	4	4	8	8	8	7	8	8	7	6	9	5	8	13	11	3	4	2	1
2	1	2	7	1	0	4	6	6	5	6	11	11	10	5	4	8	3	7	9	6	4	7	3	0
2	1	2	8	0	0	3	6	10	6	6	7	8	8	3	9	7	8	8	7	7	6	2	2	1
2	1	2	9	0	1	2	3	7	7	3	5	6	7	3	4	13	9	6	10	8	8	2	4	1
2	1	2	10	3	4	5	5	6	6	7	6	8	9	5	6	7	3	8	11	6	4	6	2	0
2	1	2	11	1	3	2	4	6	4	8	6	11	16	8	11	4	4	8	10	6	4	3	6	0
2	1	2	12	0	3	4	4	6	5	4	7	7	6	4	4	4	7	6	9	7	6	3	7	0
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2	1	2	14	0	0	5	4	6	4	5	8	8	8	3	7	6	6	7	12	8	8	2	6	1
2	1	2	15	1	0	4	3	5	12	6	7	7	7	6	7	7	9	10	7	8	6	4	5	0
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2	1	2	18	1	1	2	2	6	6	4	7	8	8	7	4	7	3	6	9	8	6	8	6	1
2	1	2	19	0	1	9	3	6	9	8	11	9	6	9	7	9	4	11	12	9	10	7	4	0
2	1	2	20	1	3	3	5	4	6	7	5	7	8	5	6	7	6	8	12	9	6	4	2	1
			Ave	0.80	1.65	3.76	4.14	5.50	6.38	8.25	6.71	7.70	8.01	5.30	6.46	6.89	5.34	7.99	10.30	7.84	5.82	4.22	4.28	0.56

Group	Patient	Treat	Loc	t0 24H	t1 96H	t2 168H	t3 264H	t4 336H	t5 432H	t6 504H	t7 576H	t8 648H	t9 744H	t10 816H	t11 912H	t12 984H	t13 1080H	t14 1152H	t15 1248H	t16 1320H	t17 1416H	t18 1488H	t19 1584H	t20 1656H
2	1	3	1	0	1	5	2	2	4	3	2	5	7	3	6	6	3	13	7	4	11	2	6	7
2	1	3	2	0	1	1	2	4	8	4	2	4	6	2	6	7	3	6	10	6	8	1	6	3
2	1	3	3	0	2	3	3	3	6	5	2	5	7	2	6	7	4	8	11	7	9	4	7	7
2	1	3	4	1	2	2	6	5	5	2	3	4	4	6	8	4	3	8	11	4	7	2	4	4
2	1	3	5	1	2	4	3	5	6	4	6	7	8	4	6	6	4	8	6	4	8	2	7	4
2	1	3	6	0	4	3	3	4	8	3	5	6	7	6	9	6	3	8	9	6	7	2	6	3
2	1	3	7	1	1	3	3	2	6	6	5	6	6	3	3	6	3	9	8	6	9	4	6	5
2	1	3	8	1	2	5	3	5	6	6	2	5	8	7	7	7	3	10	10	6	8	1	10	3
2	1	3	9	1	2	3	3	2	4	8	4	7	10	4	7	7	2	12	10	6	8	1	6	3
2	1	3	10	1	2	1	2	3	4	3	3	5	6	7	6	6	3	9	9	8	8	3	6	3
2	1	3	11	1	2	4	1	10	7	3	5	6	6	2	9	6	2	8	8	6	6	1	4	3
2	1	3	12	1	2	1	2	4	4	4	6	6	6	4	6	7	3	8	19	6	8	1	6	3
2	1	3	13	1	3	2	3	4	4	5	2	4	6	7	7	6	4	9	10	4	10	3	6	10
2	1	3	14	0	1	1	3	4	4	4	2	4	6	4	3	6	4	8	13	6	6	1	6	5
2	1	3	15	0	2	1	3	4	8	5	4	5	6	2	6	4	3	8	9	11	8	2	4	5
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2	1	3	17	2	1	2	1	5	5	3	2	3	4	4	2	6	3	5	10	6	8	2	10	4
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2	1	3	20	0	3	1	2	7	9	2	2	4	6	2	6	4	4	9	6	6	8	1	6	5
			Ave	**0.65**	**1.88**	**2.25**	**2.50**	**4.24**	**5.72**	**3.88**	**3.88**	**5.25**	**6.32**	**3.92**	**5.52**	**5.84**	**3.71**	**8.44**	**9.52**	**5.88**	**7.70**	**2.10**	**6.08**	**5.15**

Group	Patient	Treat	Loc	t0 24H	t1 96H	t2 168H	t3 264H	t4 336H	t5 432H	t6 504H	t7 576H	t8 648H	t9 744H	t10 816H	t11 912H	t12 984H	t13 1080H	t14 1152H	t15 1248H	t16 1320H	t17 1416H	t18 1488H	t19 1584H	t20 1656H
2	2	1	1	2	6	6	17	9	13	8	5	11	16	20	19	18	17	25	29	25	13	9	21	11
2	2	1	2	1	6	10	11	10	18	9	15	13	10	16	21	21	16	26	28	30	15	16	17	15
2	2	1	3	2	5	6	6	13	18	10	13	11	9	20	19	22	16	26	27	37	12	9	19	15
2	2	1	4	1	6	8	10	7	14	12	12	14	15	16	18	20	20	28	29	25	15	11	16	12
2	2	1	5	3	10	6	8	7	15	13	19	16	12	16	18	21	16	21	32	25	15	20	15	15
2	2	1	6	2	7	6	11	14	15	15	12	13	13	18	18	16	17	22	29	21	12	12	19	15
2	2	1	7	1	3	4	10	13	16	12	11	12	13	13	21	26	15	28	26	25	13	9	17	12
2	2	1	8	1	5	6	9	11	17	15	10	10	10	16	16	24	17	31	26	20	16	10	19	15
2	2	1	9	2	6	5	11	8	11	10	10	11	12	17	18	24	17	21	32	28	13	11	18	17
2	2	1	10	1	4	5	6	10	19	13	17	16	15	17	18	17	17	28	29	22	20	11	15	20
2	2	1	11	1	6	5	7	10	15	13	13	16	18	16	18	21	18	26	29	30	15	11	18	24
2	2	1	12	1	5	6	7	13	14	8	12	12	11	14	16	21	20	26	29	25	12	10	15	12
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2	2	1	20	2	6	9	9	10	16	11	14	13	12	15	19	17	18	25	29	19	15	11	18	12
			Ave	**1.45**	**5.50**	**6.29**	**9.53**	**9.75**	**15.36**	**12.13**	**12.81**	**12.80**	**12.25**	**16.00**	**18.44**	**20.93**	**16.80**	**26.32**	**29.21**	**25.42**	**15.31**	**11.41**	**18.14**	**14.70**

Group	Patient	Treat	Loc	t0 24H	t1 96H	t2 168H	t3 264H	t4 336H	t5 432H	t6 504H	t7 576H	t8 648H	t9 744H	t10 816H	t11 912H	t12 984H	t13 1080H	t14 1152H	t15 1248H	t16 1320H	t17 1416H	t18 1488H	t19 1584H	t20 1656H
2	2	2	1	2	3	8	5	10	8	12	16	13	10	19	25	13	19	25	27	29	16	9	10	11
2	2	2	2	2	9	9	7	7	12	12	16	15	13	15	17	18	20	26	20	27	22	4	15	8
2	2	2	3	2	6	11	7	13	11	11	16	16	15	20	19	19	26	21	21	26	16	7	14	6
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2	2	2	5	2	7	7	9	10	11	13	15	15	15	26	22	18	19	25	27	21	15	4	21	8
2	2	2	6	3	3	15	13	9	9	11	16	21	25	25	20	15	25	40	32	27	19	8	14	6
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2	2	2	10	0	3	6	6	7	11	10	13	14	15	24	21	18	25	22	27	25	15	7	18	9
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			Ave	1.20	5.53	8.43	7.39	10.36	11.43	12.07	16.33	15.80	14.78	18.74	21.06	17.57	24.72	25.83	27.07	27.20	15.23	7.41	14.48	7.91

Group	Patient	Treat	Loc	t0 24H	t1 96H	t2 168H	t3 264H	t4 336H	t5 432H	t6 504H	t7 576H	t8 648H	t9 744H	t10 816H	t11 912H	t12 984H	t13 1080H	t14 1152H	t15 1248H	t16 1320H	t17 1416H	t18 1488H	t19 1584H	t20 1656H
2	2	3	1	0	5	3	7	12	14	14	15	15	15	21	27	15	19	46	32	47	17	11	21	8
2	2	3	2	0	4	4	5	13	12	12	19	18	16	18	12	11	24	47	34	42	20	8	18	4
2	2	3	3	2	3	5	9	12	12	11	16	16	15	20	16	12	19	45	40	46	21	11	21	9
2	2	3	4	0	5	3	9	12	12	10	12	13	13	21	29	13	23	32	30	45	19	9	21	10
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2	2	3	6	1	6	7	6	16	17	8	20	17	14	19	18	10	27	36	31	47	24	10	21	7
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2	2	3	14	2	4	5	6	9	12	10	15	16	17	18	22	10	22	34	32	42	17	10	15	7
2	2	3	15	1	4	5	6	8	9	11	15	16	16	20	21	13	23	34	27	42	19	11	24	7
2	2	3	16	3	5	10	8	10	9	11	15	14	13	20	22	13	29	34	34	44	16	7	18	6
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2	2	3	18	0	4	4	5	13	12	20	12	13	14	17	22	12	23	38	32	30	17	9	21	10
2	2	3	19	0	2	3	6	12	9	10	19	16	12	21	18	16	20	28	28	42	19	9	26	9
2	2	3	20	2	2	6	4	12	15	9	12	12	11	20	20	13	23	29	32	42	19	6	20	8
			Ave	0.80	3.57	4.82	6.39	12.07	12.38	11.00	15.12	14.65	13.68	20.80	20.62	13.09	23.28	34.48	32.41	42.19	19.12	8.75	20.83	8.59

Group	Patient	Treat	Loc	t0 24H	t1 96H	t2 168H	t3 284H	t4 336H	t5 432H	t6 504H	t7 576H	t8 648H	t9 744H	t10 816H	t11 912H	t12 984H	t13 1080H	t14 1152H	t15 1248H	t16 1320H	t17 1416H	t18 1488H	t19 1584H	t20 1656H
2	3	1	1	4	3	10	13	13	11	9	9	11	13	6	6	8	12	10	16	20	8	8	11	9
2	3	1	2	3	7	5	7	10	8	7	12	11	10	10	15	9	7	13	16	15	12	11	13	10
2	3	1	3	3	12	6	9	9	9	9	10	10	10	11	18	15	9	6	20	11	10	9	8	8
2	3	1	4	3	6	4	3	8	11	7	3	7	11	13	16	12	6	10	13	16	13	16	3	7
2	3	1	5	4	6	10	10	17	7	8	12	14	15	13	17	11	8	13	8	13	7	15	9	8
2	3	1	6	4	3	17	8	13	10	12	6	9	11	18	9	13	9	12	16	18	12	8	11	5
2	3	1	7	2	5	7	7	4	6	6	10	10	9	9	9	6	13	13	11	11	8	9	9	6
2	3	1	8	4	5	5	5	12	7	4	2	7	12	11	9	12	8	6	15	21	6	10	9	15
2	3	1	9	4	10	3	7	10	8	11	4	8	11	12	8	7	5	13	12	10	11	9	7	8
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2	3	1	11	2	4	6	5	14	13	21	6	7	8	9	15	8	13	15	11	19	9	5	12	6
2	3	1	12	2	9	4	8	14	6	13	9	12	15	20	12	13	6	7	11	17	12	6	10	6
2	3	1	13	2	3	6	5	5	12	10	6	6	6	18	10	6	12	6	17	19	10	5	2	6
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2	3	1	16	3	6	8	10	6	9	6	6	8	10	18	8	9	8	9	17	10	8	8	6	10
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2	3	1	18	5	4	8	10	14	7	11	6	7	7	19	5	7	11	9	12	17	13	10	12	10
2	3	1	19	3	10	8	6	5	12	5	4	6	8	16	9	7	12	9	12	17	9	11	10	5
2	3	1	20	2	9	7	8	14	11	5	5	6	7	15	20	7	8	6	17	16	7	2	9	7
			Ave	3.10	6.30	7.10	7.35	10.60	8.80	8.40	6.45	8.50	10.25	13.80	11.95	8.80	9.35	9.90	13.80	15.35	9.40	8.90	8.50	7.90

Group	Patient	Treat	Loc	t0 24H	t1 96H	t2 168H	t3 284H	t4 336H	t5 432H	t6 504H	t7 576H	t8 648H	t9 744H	t10 816H	t11 912H	t12 984H	t13 1080H	t14 1152H	t15 1248H	t16 1320H	t17 1416H	t18 1488H	t19 1584H	t20 1656H
2	3	2	1	0	7	9	8	10	16	9	11	11	11	8	7	8	8	12	27	11	4	6	9	6
2	3	2	2	2	6	5	11	10	8	9	7	10	12	6	15	7	7	10	19	19	3	4	9	4
2	3	2	3	0	6	6	8	9	8	8	9	9	8	10	10	6	8	12	13	12	1	8	6	6
2	3	2	4	1	7	6	7	9	6	14	6	8	9	9	13	7	8	10	16	12	3	6	8	10
2	3	2	5	0	8	5	6	7	7	8	11	10	9	8	9	8	8	11	13	10	1	6	9	6
2	3	2	6	1	7	5	8	8	6	11	7	9	10	9	10	7	6	12	15	12	2	9	13	6
2	3	2	7	0	7	8	8	9	9	8	9	9	9	8	8	9	9	12	11	9	1	3	9	6
2	3	2	8	3	4	10	6	9	7	9	9	8	7	7	11	6	6	13	15	11	2	7	7	8
2	3	2	9	2	6	5	8	8	7	7	9	9	9	9	12	11	7	15	15	13	2	3	9	6
2	3	2	10	0	6	10	8	14	9	9	10	9	8	8	9	9	7	10	12	10	2	8	6	6
2	3	2	11	1	6	5	7	9	7	11	9	9	9	16	10	7	7	12	16	9	3	8	10	6
2	3	2	12	2	5	6	10	9	17	11	9	9	9	10	7	7	8	10	15	12	2	8	13	6
2	3	2	13	0	6	4	7	7	9	7	7	7	7	9	12	7	6	15	15	13	3	3	9	6
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2	3	2	16	0	8	6	8	6	9	11	11	10	9	8	12	6	8	11	16	15	3	4	9	6
2	3	2	17	1	6	4	8	9	8	7	7	7	7	6	10	9	6	12	10	12	2	6	6	3
2	3	2	18	3	6	4	8	7	10	9	10	11	11	8	8	6	7	11	15	16	3	6	8	11
2	3	2	19	1	6	6	15	9	9	10	5	7	8	10	9	7	7	12	15	19	3	6	13	3
2	3	2	20	1	5	5	7	9	6	9	9	9	9	8	10	4	7	16	17	9	3	3	9	4
			Ave	1.00	6.36	6.00	8.29	8.79	8.69	9.33	8.50	9.00	9.03	8.36	10.02	6.93	6.98	12.12	15.44	12.45	2.59	5.60	9.29	5.92

Group	Patient	Treat	Loc	t0 24H	t1 96H	t2 168H	t3 264H	t4 336H	t5 432H	t6 504H	t7 576H	t8 648H	t9 744H	t10 816H	t11 912H	t12 984H	t13 1080H	t14 1152H	t15 1248H	t16 1320H	t17 1416H	t18 1488H	t19 1584H	t20 1656H
2	3	1	1	4	3	10	13	13	11	9	9	11	13	6	6	8	12	10	16	20	8	8	11	9
2	3	1	2	3	7	5	7	10	8	7	12	11	10	10	15	9	7	13	16	15	12	11	13	10
2	3	1	3	3	12	6	9	9	9	9	10	10	10	11	18	15	9	6	20	11	10	9	8	8
2	3	1	4	3	8	4	3	8	11	7	3	7	11	13	16	12	6	10	13	16	13	16	3	7
2	3	1	5	4	6	10	10	17	7	8	12	14	15	13	17	11	8	13	8	13	7	15	9	8
2	3	1	6	4	3	17	8	13	10	12	6	9	11	18	9	13	9	12	16	18	12	8	11	5
2	3	1	7	2	5	7	7	4	6	6	10	10	9	9	9	6	13	13	11	11	8	9	9	6
2	3	1	8	4	5	5	5	12	7	4	2	7	12	11	9	12	8	6	15	21	6	10	9	15
2	3	1	9	4	10	3	7	10	8	11	4	8	11	12	8	7	5	13	12	10	11	9	7	8
2	3	1	10	4	8	6	5	9	7	6	6	9	12	15	16	8	9	5	10	11	9	15	6	6
2	3	1	11	2	4	6	5	14	13	21	6	7	8	9	15	8	13	15	11	19	9	5	12	6
2	3	1	12	2	9	4	8	14	6	13	9	12	15	20	12	13	6	7	11	17	12	6	10	6
2	3	1	13	2	3	6	5	5	12	10	6	6	6	18	10	6	12	6	17	19	10	5	2	6
2	3	1	14	3	3	8	6	12	6	5	3	8	13	20	10	5	7	10	17	15	7	7	5	8
2	3	1	15	1	9	7	6	16	7	7	3	5	6	10	8	5	12	11	16	13	6	9	11	8
2	3	1	16	3	6	8	10	6	9	6	6	8	10	18	8	9	8	9	17	10	8	8	6	10
2	3	1	17	4	2	7	9	11	9	6	7	9	11	13	19	8	12	15	9	18	11	5	7	10
2	3	1	18	5	4	8	10	14	7	11	6	7	7	19	5	7	11	9	12	17	13	10	12	10
2	3	1	19	3	10	8	6	5	12	5	4	6	8	16	9	7	12	9	12	17	9	11	10	5
2	3	1	20	2	9	7	8	14	11	5	5	6	7	15	20	7	8	6	17	16	7	2	9	7
			Ave	3.10	6.30	7.10	7.35	10.80	8.80	8.40	6.45	8.50	10.25	13.80	11.95	8.80	9.35	9.90	13.80	15.35	9.40	8.90	8.50	7.90

Group	Patient	Treat	Loc	t0 24H	t1 96H	t2 168H	t3 264H	t4 336H	t5 432H	t6 504H	t7 576H	t8 648H	t9 744H	t10 816H	t11 912H	t12 984H	t13 1080H	t14 1152H	t15 1248H	t16 1320H	t17 1416H	t18 1488H	t19 1584H	t20 1656H
2	3	2	1	0	7	9	8	10	16	9	11	11	11	8	7	8	8	12	27	11	4	6	9	6
2	3	2	2	2	6	5	11	10	8	9	7	10	12	6	15	7	7	10	19	19	3	4	9	4
2	3	2	3	0	6	6	8	9	8	8	9	9	8	10	10	6	8	12	13	12	1	8	6	6
2	3	2	4	1	7	6	7	9	6	14	6	8	9	9	13	7	8	10	16	12	3	6	8	10
2	3	2	5	0	8	5	6	7	7	8	11	10	9	8	9	8	8	11	13	10	1	6	9	6
2	3	2	6	1	7	5	8	8	6	11	7	9	10	9	10	7	6	12	15	12	2	9	13	6
2	3	2	7	0	7	8	8	9	9	8	9	9	9	8	8	9	9	12	11	9	1	3	9	6
2	3	2	8	3	4	10	6	9	7	9	9	8	7	7	11	6	6	13	15	11	2	7	7	8
2	3	2	9	2	6	5	8	8	7	7	9	9	9	9	12	11	7	15	15	13	2	3	9	6
2	3	2	10	0	6	10	8	14	9	9	10	9	8	8	9	9	7	10	12	10	2	8	6	6
2	3	2	11	1	6	5	7	9	7	11	9	9	9	16	10	7	7	12	16	9	3	8	10	6
2	3	2	12	2	5	6	10	9	17	11	9	9	9	10	7	7	8	10	15	12	2	8	13	6
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2	3	2	16	0	8	6	8	6	9	11	11	10	9	8	12	6	8	11	16	15	3	4	9	6
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2	3	2	18	3	6	4	8	7	10	9	10	11	11	8	8	6	7	11	15	16	3	6	8	11
2	3	2	19	1	6	6	15	9	9	10	5	7	8	10	9	7	7	12	15	19	3	6	13	3
2	3	2	20	1	5	5	7	9	6	9	9	9	9	8	10	4	7	16	17	9	3	3	9	4
			Ave	1.00	6.36	6.00	8.29	8.79	8.69	9.33	8.50	9.00	9.03	8.36	10.02	6.93	6.98	12.12	15.44	12.45	2.59	5.60	9.29	5.92

Group	Patient	Treat	Loc	t0 24H	t1 96H	t2 168H	t3 264H	t4 336H	t5 432H	t6 504H	t7 576H	t8 648H	t9 744H	t10 816H	t11 912H	t12 984H	t13 1080H	t14 1152H	t15 1248H	t16 1320H	t17 1416H	t18 1488H	t19 1584H	t20 1656H
2	4	2	1	1	6	2	2	9	1	7	9	8	8	25	8	5	10	13	6	9	10	8	7	4
2	4	2	2	0	8	3	4	6	1	7	12	9	9	19	11	10	8	11	6	8	8	8	7	10
2	4	2	3	1	8	4	3	5	0	7	9	6	8	9	6	6	11	9	10	5	8	7	7	2
2	4	2	4	0	7	4	3	3	2	5	9	8	8	8	9	5	6	9	9	10	8	6	5	2
2	4	2	5	2	5	4	2	5	0	11	13	5	9	11	7	5	9	8	9	8	7	6	7	7
2	4	2	6	1	4	2	3	5	0	5	9	5	8	18	7	6	12	10	8	8	7	6	6	6
2	4	2	7	3	5	2	3	7	1	4	9	7	12	12	6	4	12	8	10	10	13	6	8	8
2	4	2	8	1	3	3	2	6	2	10	7	8	12	17	6	4	13	10	8	9	9	8	7	3
2	4	2	9	0	8	5	4	4	0	4	9	8	6	13	5	10	12	10	15	12	9	4	4	7
2	4	2	10	1	6	3	3	3	0	5	9	7	9	12	7	5	14	10	15	8	12	6	7	10
2	4	2	11	2	5	2	2	3	2	9	9	11	10	14	6	11	8	10	9	5	7	5	8	1
2	4	2	12	1	3	4	1	5	0	12	10	15	14	21	5	6	10	11	7	8	7	6	7	9
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2	4	2	15	1	5	3	3	5	3	4	14	6	8	14	5	5	7	8	7	11	6	6	7	7
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2	4	2	20	4	5	3	5	5	1	7	6	8	7	14	8	6	10	10	9	5	10	7	8	3
			Ave	1.65	5.44	3.47	2.59	5.31	1.00	6.80	8.93	7.56	8.63	13.93	6.82	6.25	9.56	9.69	8.76	8.13	8.79	6.29	7.29	5.40

Group	Patient	Treat	Loc	t0 24H	t1 96H	t2 168H	t3 264H	t4 336H	t5 432H	t6 504H	t7 576H	t8 648H	t9 744H	t10 816H	t11 912H	t12 984H	t13 1080H	t14 1152H	t15 1248H	t16 1320H	t17 1416H	t18 1488H	t19 1584H	t20 1656H
2	4	3	1	3	5	10	3	8	1	3	7	9	10	6	10	8	7	10	16	10	9	7	7	8
2	4	3	2	3	5	12	4	3	2	6	14	15	7	9	15	10	9	10	17	6	11	8	8	5
2	4	3	3	1	6	3	5	4	1	6	10	8	10	9	9	9	8	10	17	6	9	6	8	5
2	4	3	4	5	6	4	3	4	0	3	9	6	11	9	14	8	5	10	15	8	9	5	10	3
2	4	3	5	2	8	3	4	7	2	7	7	8	9	7	8	5	12	12	15	6	9	10	7	11
2	4	3	6	2	8	4	6	3	1	4	11	10	8	9	12	5	8	10	17	6	5	5	11	7
2	4	3	7	2	8	3	3	6	2	3	9	4	7	9	8	8	8	7	15	5	6	12	8	11
2	4	3	8	1	8	3	5	6	2	8	10	8	10	16	12	13	5	7	11	6	9	8	8	8
2	4	3	9	2	5	5	3	6	1	4	6	13	10	9	15	8	6	7	12	7	9	7	11	4
2	4	3	10	2	8	6	3	6	1	5	7	6	9	8	9	6	5	10	10	5	9	8	5	4
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2	4	3	12	3	10	4	2	6	0	5	11	7	11	10	9	9	6	17	14	7	9	5	9	9
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2	4	3	18	5	6	4	3	3	1	6	9	5	8	9	10	10	8	10	13	9	7	6	8	9
2	4	3	19	2	6	3	3	6	0	4	8	8	11	10	9	9	8	8	14	9	7	8	8	6
2	4	3	20	6	6	2	3	4	0	5	6	5	13	8	11	6	11	7	14	8	9	8	6	14
			Ave	2.70	7.53	4.33	3.56	5.69	1.30	5.22	9.25	7.50	10.06	9.33	10.50	7.63	7.53	10.38	13.69	7.25	8.71	7.50	8.33	7.00

Group	Patient	Treat	Loc	t0 24H	t1 96H	t2 168H	t3 264H	t4 336H	t5 432H	t6 504H	t7 576H	t8 648H	t9 744H	t10 816H	t11 912H	t12 984H	t13 1080H	t14 1152H	t15 1248H	t16 1320H	t17 1416H	t18 1488H	t19 1584H	t20 1656H
2	3	3	1	3	3	3	7	7	10	10	10	11	11	11	12	7	7	25	18	12	3	7	8	9
2	3	3	2	3	5	3	13	8	10	10	10	10	10	8	13	8	11	19	20	12	2	9	8	6
2	3	3	3	1	3	4	9	8	10	10	14	12	9	11	13	7	10	18	18	17	6	7	8	7
2	3	3	4	0	3	5	11	5	11	10	11	11	10	11	10	8	7	18	18	13	4	7	8	8
2	3	3	5	2	2	3	9	8	7	10	9	9	8	10	9	7	8	18	13	13	2	10	8	7
2	3	3	6	3	3	6	10	8	10	12	13	12	11	16	18	7	8	18	19	17	4	7	6	6
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2	3	3	20	3	6	3	7	6	12	9	16	13	10	10	13	6	11	13	15	17	3	7	11	10
			Ave	2.00	3.45	4.28	8.88	7.87	9.56	10.38	11.00	10.75	9.94	10.67	12.95	7.84	8.17	17.63	17.68	16.65	3.61	7.28	8.03	6.48

Group	Patient	Treat	Loc	t0 24H	t1 96H	t2 168H	t3 264H	t4 336H	t5 432H	t6 504H	t7 576H	t8 648H	t9 744H	t10 616H	t11 912H	t12 984H	t13 1080H	t14 1152H	t15 1248H	t16 1320H	t17 1416H	t18 1488H	t19 1584H	t20 1656H
2	4	1	1	2	3	2	4	2	2	3	15	5	6	8	25	3	7	7	8	6	5	12	5	12
2	4	1	2	1	3	5	2	2	1	5	11	8	9	10	15	4	8	11	11	7	6	6	5	15
2	4	1	3	1	2	7	4	4	3	4	8	8	6	11	9	4	5	10	6	9	7	6	6	7
2	4	1	4	2	2	5	4	5	0	6	9	8	7	6	11	5	7	8	7	6	6	6	5	6
2	4	1	5	2	3	2	2	3	3	9	5	4	7	9	13	5	9	9	6	8	10	6	4	9
2	4	1	6	1	3	5	3	3	2	4	7	5	12	11	13	8	6	12	11	7	6	6	6	6
2	4	1	7	3	5	5	6	3	2	3	9	8	9	6	13	4	8	8	8	8	8	7	7	9
2	4	1	8	1	3	5	4	3	1	8	8	4	11	9	13	6	10	10	8	3	5	8	5	8
2	4	1	9	1	6	3	3	3	2	5	13	8	9	9	13	6	6	6	6	4	7	6	6	11
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2	4	1	11	2	2	5	11	2	1	4	5	6	7	9	12	6	12	12	10	4	10	3	6	10
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2	4	1	16	3	1	3	2	7	0	6	8	11	9	9	7	3	7	9	8	6	5	9	9	6
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			Ave	2.10	3.00	4.71	4.47	3.44	1.50	5.00	8.20	7.53	8.50	8.75	12.07	4.89	7.56	9.14	7.71	5.50	7.13	5.67	5.80	9.33

Group	Patient	Treat	Loc	t0 24H	t1 96H	t2 168H	t3 264H	t4 336H	t5 432H	t6 504H	t7 576H	t8 648H	t9 744H	t10 816H	t11 912H	t12 984H	t13 1080H	t14 1152H	t15 1248H	t16 1320H	t17 1416H	t18 1488H	t19 1584H	t20 1656H
2	5	1	1	1	9	7	9	13	15	10	10	13	16	20	19	22	17	28	31	24	16	26	20	19
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2	5	1	3	0	5	10	5	12	15	13	13	14	15	15	16	17	16	27	24	20	16	22	26	18
2	5	1	4	2	6	5	11	18	15	13	13	12	11	15	19	17	16	27	24	19	16	26	26	15
2	5	1	5	2	6	7	6	8	11	11	11	13	15	12	21	15	13	26	24	24	21	26	21	18
2	5	1	6	0	8	6	8	13	14	12	13	15	17	15	19	24	15	27	25	17	22	35	25	16
2	5	1	7	3	6	7	5	10	11	11	15	17	19	17	21	15	15	27	21	21	19	27	22	21
2	5	1	8	2	7	7	15	7	15	13	12	14	15	13	13	18	16	25	24	25	18	26	20	17
2	5	1	9	4	8	9	11	10	11	17	11	15	18	11	15	18	18	27	24	27	22	22	22	15
2	5	1	10	2	6	7	5	7	14	10	13	12	11	12	19	13	17	25	24	21	19	25	21	16
2	5	1	11	0	4	5	10	10	22	9	10	13	15	15	15	18	16	31	24	29	18	28	19	20
2	5	1	12	2	4	5	11	8	18	11	9	11	12	13	19	18	16	27	20	18	16	25	22	15
2	5	1	13	3	5	8	8	10	17	15	18	17	15	17	18	15	18	22	21	18	18	30	19	18
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2	5	1	16	0	4	7	10	10	15	10	11	12	12	15	21	15	16	28	24	21	25	31	19	21
2	5	1	17	4	6	7	8	8	13	9	10	15	19	15	22	18	12	27	21	18	18	27	22	20
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2	5	1	20	1	5	8	5	14	15	10	13	14	15	16	20	18	12	24	24	18	16	21	21	19
			Ave	1.60	5.73	7.11	8.19	10.36	14.94	11.24	12.57	13.90	14.56	14.82	16.56	17.60	16.11	26.57	23.71	20.72	18.38	26.24	21.76	17.85

Group	Patient	Treat	Loc	t0 24H	t1 96H	t2 168H	t3 264H	t4 336H	t5 432H	t6 504H	t7 576H	t8 648H	t9 744H	t10 816H	t11 912H	t12 984H	t13 1080H	t14 1152H	t15 1248H	t16 1320H	t17 1416H	t18 1488H	t19 1584H	t20 1656H
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2	5	2	2	3	8	8	12	17	14	16	17	17	17	25	18	15	15	20	22	15	8	15	7	9
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			Ave	2.20	6.80	8.13	11.87	14.40	16.44	14.25	17.75	17.35	16.80	20.48	20.96	17.92	16.73	20.23	22.05	15.23	8.96	15.82	10.20	8.06

Group	Patient	Treat	Loc	t0 24H	t1 96H	t2 168H	t3 264H	t4 336H	t5 432H	t6 504H	t7 576H	t8 648H	t9 744H	t10 816H	t11 912H	t12 984H	t13 1080H	t14 1152H	t15 1248H	t16 1320H	t17 1416H	t18 1488H	t19 1584H	t20 1656H
2	5	1	1	1	9	7	9	13	15	10	10	13	16	20	19	22	17	28	31	24	16	26	20	19
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			Ave	1.80	5.73	7.11	8.19	10.36	14.94	11.24	12.57	13.90	14.58	14.82	18.56	17.60	16.11	26.57	23.71	20.72	18.38	26.24	21.76	17.85

Group	Patient	Treat	Loc	t0 24H	t1 96H	t2 168H	t3 264H	t4 336H	t5 432H	t6 504H	t7 576H	t8 648H	t9 744H	t10 816H	t11 912H	t12 984H	t13 1080H	t14 1152H	t15 1248H	t16 1320H	t17 1416H	t18 1488H	t19 1584H	t20 1656H
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			Ave	2.20	6.80	8.13	11.87	14.40	18.44	14.25	17.75	17.35	16.80	20.48	20.96	17.92	16.73	20.23	22.05	15.23	8.96	15.82	10.20	8.06

Group	Patient	Treat	Loc	t0 24H	t1 96H	t2 168H	t3 264H	t4 336H	t5 432H	t6 504H	t7 576H	t8 648H	t9 744H	t10 816H	t11 912H	t12 984H	t13 1080H	t14 1152H	t15 1248H	t16 1320H	t17 1416H	t18 1488H	t19 1584H	t20 1656H
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2	5	3	19	2	2	7	9	12	9	10	14	15	16	20	25	18	12	16	20	25	7	14	13	16
2	5	3	20	1	6	6	10	12	15	9	16	16	15	18	20	18	24	22	20	16	10	15	12	12
			Ave	1.35	3.67	6.71	8.88	9.58	12.47	10.44	16.07	15.35	14.00	15.28	22.03	18.97	16.50	21.50	20.37	19.86	8.96	14.02	13.36	16.24

Group	Patient	Treat	Loc	t0 24H	t1 96H	t2 168H	t3 264H	t4 336H	t5 432H	t6 504H	t7 576H	t8 648H	t9 744H	t10 816H	t11 912H	t12 984H	t13 1080H	t14 1152H	t15 1248H	t16 1320H	t17 1416H	t18 1488H	t19 1584H	t20 1656H
3	1	1	1	1	3	3	6	11	5	5	10	9	8	10	11	8	10	7	12	6	8	12	8	12
3	1	1	2	1	2	3	6	5	8	4	5	7	9	7	9	7	11	9	13	8	6	10	9	11
3	1	1	3	1	4	4	8	5	5	8	5	7	8	9	12	12	17	10	13	7	9	12	8	7
3	1	1	4	1	3	4	5	6	11	5	4	6	8	11	8	12	9	8	7	7	11	12	9	7
3	1	1	5	2	3	6	6	7	7	10	5	7	8	13	8	8	9	7	8	9	7	9	10	11
3	1	1	6	1	4	3	5	5	6	11	5	6	6	9	6	7	12	13	8	7	7	11	6	9
3	1	1	7	2	6	2	6	10	7	5	5	7	9	8	6	11	10	7	7	8	9	12	6	7
3	1	1	8	3	4	6	6	7	7	6	3	6	8	8	12	10	12	15	8	4	9	12	6	12
3	1	1	9	2	6	3	3	8	7	5	3	6	8	9	8	10	9	9	9	6	7	12	9	10
3	1	1	10	2	2	2	6	4	8	7	3	5	7	9	13	10	9	11	7	4	8	10	8	10
3	1	1	11	3	2	4	8	6	5	6	10	9	8	10	6	11	10	8	10	4	9	13	8	10
3	1	1	12	0	5	4	4	5	5	9	6	8	10	9	6	10	8	13	11	11	13	9	6	9
3	1	1	13	1	6	3	5	10	6	5	6	7	8	8	6	8	9	9	12	7	12	11	8	7
3	1	1	14	1	2	2	4	9	9	6	9	10	10	6	8	15	10	8	10	7	10	11	9	11
3	1	1	15	1	4	2	4	7	7	6	6	7	8	11	9	11	7	12	9	6	9	11	8	8
3	1	1	16	1	3	5	4	6	8	4	5	7	8	6	8	10	7	10	11	4	9	13	7	9
3	1	1	17	0	3	3	5	7	5	5	6	6	6	9	9	10	11	9	18	4	10	15	10	9
3	1	1	19	1	3	4	5	4	5	4	4	6	8	9	7	12	10	6	10	6	7	9	8	9
3	1	1	20	0	5	4	3	7	8	6	3	5	6	9	11	8	10	7	10	4	12	15	7	12
			Ave	1.20	3.61	3.49	5.25	6.73	6.75	6.16	5.44	6.89	7.72	8.88	8.44	10.01	10.02	9.34	10.21	6.31	9.10	11.42	7.60	9.44

Group	Patient	Treat	Loc	t0 24H	t1 96H	t2 168H	t3 264H	t4 336H	t5 432H	t6 504H	t7 576H	t8 648H	t9 744H	t10 816H	t11 912H	t12 984H	t13 1080H	t14 1152H	t15 1248H	t16 1320H	t17 1416H	t18 1488H	t19 1584H	t20 1656H
3	1	2	1	3	6	4	9	11	13	5	10	9	8	7	7	8	15	8	24	9	6	8	6	2
3	1	2	2	4	4	5	4	7	9	7	5	6	7	6	7	11	9	9	10	10	6	7	9	1
3	1	2	3	2	4	4	4	5	10	7	7	7	7	10	6	6	7	8	12	12	6	17	5	3
3	1	2	4	3	4	6	3	9	10	6	7	7	7	8	9	9	9	10	16	6	6	8	5	0
3	1	2	5	4	4	3	4	8	11	8	6	7	7	4	7	10	8	12	11	8	3	8	5	1
3	1	2	6	3	4	5	3	7	9	7	9	8	6	4	8	8	13	11	16	11	6	9	5	0
3	1	2	7	1	4	4	4	7	9	5	5	6	8	6	7	6	7	7	13	8	9	7	2	2
3	1	2	8	1	10	3	5	5	11	4	8	10	11	6	7	13	10	12	10	9	9	13	4	2
3	1	2	9	2	2	4	8	6	11	6	5	6	6	7	8	8	16	10	13	7	7	10	6	1
3	1	2	10	0	7	6	5	6	9	6	11	9	7	7	6	8	10	11	11	6	8	7	3	0
3	1	2	11	2	5	6	6	6	8	5	6	7	7	7	8	6	8	8	9	7	7	10	2	3
3	1	2	12	0	2	4	5	7	13	8	7	7	7	7	7	6	9	13	11	7	7	7	3	2
3	1	2	13	1	3	4	4	7	9	6	8	8	7	6	7	6	8	9	11	12	3	13	3	0
3	1	2	14	1	3	4	5	6	10	4	6	6	6	4	7	8	9	8	9	8	6	15	9	2
3	1	2	15	0	3	4	8	6	10	5	7	7	7	9	6	8	7	12	13	6	4	10	2	0
3	1	2	16	2	4	7	6	12	11	4	6	7	7	7	10	8	9	10	13	7	4	20	4	1
3	1	2	17	1	2	7	5	7	10	4	8	8	7	9	7	7	9	9	13	6	6	8	4	1
3	1	2	18	2	3	6	5	7	7	4	7	7	6	7	9	9	10	7	13	8	6	10	8	0
3	1	2	19	1	2	5	3	6	9	11	8	7	6	4	7	11	10	6	10	9	3	10	3	0
3	1	2	20	2	4	7	6	7	10	5	7	7	6	8	7	6	7	7	17	10	7	7	6	0
			Ave	1.75	4.00	4.69	5.11	7.13	9.93	5.64	7.20	7.30	6.72	6.52	7.32	7.96	9.49	9.37	12.69	8.21	5.92	10.14	4.76	1.18

Group	Patient	Treat	Loc	t0 24H	t1 96H	t2 168H	t3 264H	t4 336H	t5 432H	t6 504H	t7 576H	t8 648H	t9 744H	t10 816H	t11 912H	t12 984H	t13 1080H	t14 1152H	t15 1248H	t16 1320H	t17 1416H	t18 1488H	t19 1584H	t20 1656H
3	1	3	1	1	3	4	4	7	11	7	5	6	7	9	13	9	5	17	21	7	3	7	2	8
3	1	3	2	1	2	3	5	7	9	5	4	8	11	4	8	9	10	17	11	8	6	5	6	8
3	1	3	3	2	2	7	5	4	7	5	4	6	7	7	8	6	6	15	15	7	3	5	2	8
3	1	3	4	2	2	4	5	6	11	7	3	5	6	9	9	9	4	15	19	9	3	6	6	2
3	1	3	5	1	4	4	4	7	5	6	4	5	6	7	9	9	6	12	13	11	4	7	2	2
3	1	3	6	1	7	6	6	6	11	9	5	8	10	6	11	9	10	10	10	7	5	7	3	8
3	1	3	7	1	3	4	5	7	8	7	6	6	6	8	6	7	7	13	12	8	4	6	2	3
3	1	3	8	2	6	4	8	8	8	5	5	7	8	4	9	8	8	15	13	8	6	7	6	3
3	1	3	9	2	4	4	3	6	8	19	4	4	4	4	12	9	6	11	11	10	5	5	4	2
3	1	3	10	0	3	7	4	6	5	7	4	6	7	7	9	7	7	17	13	8	4	3	4	8
3	1	3	11	1	3	8	4	6	5	7	4	6	7	11	6	12	13	9	13	11	4	3	3	7
3	1	3	12	3	3	4	5	12	8	5	5	6	7	2	6	9	10	13	15	6	6	3	2	5
3	1	3	13	1	4	3	5	7	6	5	4	6	7	9	8	9	10	13	9	8	4	5	6	3
3	1	3	14	2	4	4	4	13	11	6	5	5	4	7	9	10	11	11	15	8	5	3	3	3
3	1	3	15	0	4	3	8	6	8	4	5	6	7	7	8	8	8	13	10	10	4	3	6	5
3	1	3	16	0	4	3	5	6	10	6	4	6	8	7	9	15	16	13	11	8	5	4	3	3
3	1	3	17	3	5	6	4	8	8	8	8	10	12	7	10	9	10	10	13	13	8	5	3	3
3	1	3	18	2	4	2	3	8	7	6	5	6	6	6	7	15	16	15	13	8	7	4	3	4
3	1	3	19	2	3	4	5	5	7	8	6	7	7	9	10	8	8	10	13	7	6	5	6	6
3	1	3	20	1	3	2	5	7	6	7	6	5	4	4	9	11	12	13	9	6	4	5	4	6
			Ave	1.40	3.56	4.40	4.60	7.13	7.94	6.93	4.75	6.20	7.00	6.65	8.68	9.38	9.15	13.07	13.02	8.44	4.90	4.91	3.86	4.76

Group	Patient	Treat	Loc	t0 24H	t1 96H	t2 168H	t3 264H	t4 336H	t5 432H	t6 504H	t7 576H	t8 648H	t9 744H	t10 816H	t11 912H	t12 984H	t13 1080H	t14 1152H	t15 1248H	t16 1320H	t17 1416H	t18 1488H	t19 1584H	t20 1656H
3	2	1	1	0	1	0	0	0	2	2	5	2	10	6	5	3	2	5	5	3	9	10	10	6
3	2	1	2	0	1	3	1	2	2	4	4	2	3	5	11	6	5	7	3	3	6	10	10	5
3	2	1	3	2	4	0	2	1	3	2	1	1	5	9	4	6	4	8	4	5	6	10	10	5
3	2	1	4	1	3	2	0	2	1	3	2	4	8	4	7	3	4	7	3	5	5	11	6	6
3	2	1	5	0	1	1	0	0	2	1	1	4	7	8	3	5	5	7	5	9	4	11	7	5
3	2	1	6	0	3	1	0	1	2	1	2	3	3	5	8	8	4	7	4	4	3	14	8	3
3	2	1	7	2	1	2	0	0	4	2	1	3	5	5	7	2	9	7	5	3	6	7	10	7
3	2	1	8	2	3	1	0	3	3	4	2	2	9	5	6	8	7	7	5	4	8	10	8	5
3	2	1	9	0	4	0	0	0	2	1	1	2	3	6	6	4	4	5	4	4	5	11	12	7
3	2	1	10	0	4	1	0	1	1	1	2	3	6	8	5	4	2	10	3	4	8	10	8	6
3	2	1	11	1	5	0	0	0	2	0	2	1	4	9	7	7	3	5	6	6	8	12	6	6
3	2	1	12	2	1	1	0	2	3	0	1	6	7	7	6	4	4	6	5	3	6	9	10	3
3	2	1	13	0	3	0	0	2	4	1	1	1	6	4	6	2	4	4	7	5	6	10	11	3
3	2	1	14	3	2	0	0	1	2	0	2	3	3	4	8	3	2	6	8	5	4	9	6	4
3	2	1	15	0	1	0	0	0	1	1	3	2	9	4	5	4	4	7	5	5	5	8	8	5
3	2	1	16	3	3	2	0	0	3	0	1	1	7	4	10	4	2	6	4	6	3	12	8	5
3	2	1	17	1	1	1	0	4	2	0	3	3	6	6	6	5	2	10	3	8	4	8	7	3
3	2	1	18	1	2	2	0	0	2	2	2	2	3	6	6	2	2	8	10	5	8	7	7	5
3	2	1	19	0	3	1	0	0	1	1	1	4	6	4	6	2	2	8	4	6	3	10	8	4
3	2	1	20	2	1	1	0	0	5	1	3	3	6	4	5	3	4	6	13	3	8	10	6	4
			Ave	1.00	2.37	0.95	0.15	0.95	2.41	1.35	2.00	2.58	5.75	5.61	8.41	4.28	3.69	8.73	5.38	4.75	5.71	9.79	8.40	4.81

Group	Patient	Treat	Loc	t0 24H	t1 96H	t2 168H	t3 264H	t4 336H	t5 432H	t6 504H	t7 576H	t8 648H	t9 744H	t10 816H	t11 912H	t12 984H	t13 1080H	t14 1152H	t15 1248H	t16 1320H	t17 1416H	t18 1488H	t19 1584H	t20 1658H
3	2	2	1	5	6	4	1	4	8	6	5	6	8	5	10	10	3	7	8	7	7	10	8	7
3	2	2	2	1	4	4	1	4	7	3	5	5	7	8	12	8	4	11	7	9	5	8	7	10
3	2	2	3	4	4	2	3	4	7	7	5	3	10	9	11	13	5	6	11	8	5	12	14	6
3	2	2	4	1	4	5	4	4	5	4	4	4	11	8	16	13	3	4	11	6	8	5	9	4
3	2	2	5	0	3	3	2	4	5	3	7	6	7	8	14	10	3	7	7	9	8	8	7	8
3	2	2	6	4	2	3	3	4	5	4	3	4	5	8	11	11	4	12	8	7	5	8	8	7
3	2	2	7	2	3	3	3	3	5	3	11	5	8	8	17	13	7	6	8	7	13	9	13	5
3	2	2	8	1	4	4	1	7	4	3	5	7	5	8	14	13	6	7	6	9	6	8	11	6
3	2	2	9	4	4	1	3	2	4	5	6	5	8	6	13	13	2	7	6	8	7	6	9	6
3	2	2	10	3	7	2	4	2	5	7	5	5	9	8	12	13	4	8	6	9	7	8	7	8
3	2	2	11	1	4	2	3	4	5	9	4	3	8	8	13	12	3	8	14	9	9	6	14	16	9
3	2	2	12	3	4	1	5	4	3	5	3	4	11	7	16	12	3	5	9	9	6	14	16	9
3	2	2	13	2	4	3	1	3	4	15	5	7	9	8	19	14	5	5	5	9	6	8	7	7
3	2	2	14	3	2	3	3	7	3	5	6	5	8	10	9	17	5	7	9	12	9	8	8	7
3	2	2	15	3	6	2	3	6	3	4	3	3	7	8	7	22	5	5	6	10	7	8	8	5
3	2	2	16	1	2	3	3	2	5	8	6	4	5	8	14	10	7	7	8	8	7	6	7	6
3	2	2	17	0	2	3	4	2	5	4	5	7	6	6	12	13	2	9	10	8	10	6	8	5
3	2	2	18	3	2	2	1	5	4	6	5	3	10	8	12	10	3	13	7	8	6	8	10	5
3	2	2	19	4	5	1	2	2	4	5	4	3	8	8	11	14	4	6	8	11	7	8	7	4
3	2	2	20	1	4	2	2	3	5	3	3	7	6	7	9	17	2	5	6	11	10	7	10	5
			Ave	2.30	3.73	2.63	2.53	3.76	4.73	5.47	5.00	4.76	7.73	7.57	12.60	12.85	4.00	7.29	8.00	8.60	7.24	8.13	9.17	8.35

Group	Patient	Treat	Loc	t0 24H	t1 96H	t2 168H	t3 264H	t4 336H	t5 432H	t6 504H	t7 576H	t8 648H	t9 744H	t10 816H	t11 912H	t12 984H	t13 1080H	t14 1152H	t15 1248H	t16 1320H	t17 1416H	t18 1488H	t19 1584H	t20 1656H
3	2	3	1	5	4	10	4	2	4	10	2	5	9	10	9	8	5	10	8	9	9	9	13	5
3	2	3	2	1	8	3	1	2	4	7	3	2	6	12	13	10	5	7	6	3	4	9	12	13
3	2	3	3	4	6	3	1	3	2	8	2	5	8	6	10	9	4	8	5	13	6	6	10	4
3	2	3	4	6	7	6	3	6	4	3	6	2	10	9	10	9	7	7	12	4	3	13	9	6
3	2	3	5	3	6	3	5	5	6	13	4	6	7	11	9	9	9	11	9	6	6	6	9	7
3	2	3	6	1	2	4	1	3	6	5	4	3	6	6	9	9	5	6	7	3	3	9	6	7
3	2	3	7	3	3	6	2	2	4	5	4	5	12	10	7	10	4	8	6	5	5	12	10	11
3	2	3	8	3	2	3	2	4	4	9	8	4	12	8	9	8	5	8	5	5	3	9	9	7
3	2	3	9	4	4	4	2	4	3	5	3	5	10	7	9	6	4	6	4	4	5	15	10	10
3	2	3	10	3	3	7	2	3	4	6	4	4	9	10	7	8	6	6	6	6	9	14	6	6
3	2	3	11	3	2	5	2	3	6	3	4	4	17	8	9	12	6	8	5	6	6	7	6	7
3	2	3	12	0	4	5	2	3	5	3	6	2	13	8	12	9	3	8	5	4	4	9	8	8
3	2	3	13	4	5	6	2	2	3	4	3	3	9	9	9	7	4	11	5	3	4	13	7	6
3	2	3	14	3	2	3	1	6	3	6	4	3	7	8	9	8	3	11	4	3	6	7	6	6
3	2	3	15	4	4	3	3	4	5	4	2	2	7	8	6	9	5	8	6	6	8	7	8	5
3	2	3	16	2	6	5	3	4	2	6	4	2	9	9	10	7	4	7	6	4	3	7	8	4
3	2	3	17	4	3	5	2	3	7	6	2	7	7	8	6	10	5	6	6	6	7	8	8	6
3	2	3	18	4	3	4	2	3	3	5	4	4	8	8	10	9	6	8	4	11	5	7	9	7
3	2	3	19	2	2	4	4	2	4	6	7	4	9	8	9	9	4	5	8	3	4	11	8	7
3	2	3	20	4	2	3	2	4	4	3	2	6	10	10	6	13	10	6	5	7	7	9	10	4
			Ave	3.15	3.89	4.53	2.33	3.47	4.19	5.82	3.88	3.88	9.31	8.61	8.88	8.93	5.24	7.57	6.12	5.50	5.39	9.47	8.53	6.76

Group	Patient	Treat	Loc	t0 24H	t1 96H	t2 168H	t3 264H	t4 336H	t5 432H	t6 504H	t7 576H	t8 648H	t9 744H	t10 816H	t11 912H	t12 984H	t13 1080H	t14 1152H	t15 1248H	t16 1320H	t17 1416H	t18 1488H	t19 1584H	t20 1656H
3	3	1	1	2	4	9	4	8	6	11	8	7	6	8	10	9	9	9	17	10	8	12	7	17
3	3	1	2	1	5	3	5	6	7	11	11	10	8	11	10	10	12	10	10	12	7	9	7	19
3	3	1	3	0	6	3	3	7	10	7	7	8	8	12	10	9	7	12	8	12	9	13	10	17
3	3	1	4	1	4	5	5	5	10	9	5	7	9	8	7	8	13	9	9	12	11	12	11	12
3	3	1	5	0	4	5	6	6	8	9	8	10	12	7	9	11	7	11	11	13	6	9	13	15
3	3	1	6	2	5	4	3	8	8	8	5	7	8	7	11	7	10	9	11	13	6	19	11	12
3	3	1	7	1	5	5	4	8	6	9	6	7	8	8	12	8	7	11	11	9	9	13	10	15
3	3	1	8	1	4	5	5	7	6	14	8	10	11	10	16	9	16	9	11	13	9	17	9	12
3	3	1	9	1	3	7	4	5	8	9	5	7	8	8	11	9	8	8	11	12	12	20	8	15
3	3	1	10	0	7	4	5	6	9	8	5	9	12	8	8	7	15	11	9	12	9	13	12	15
3	3	1	11	3	6	6	5	6	11	7	9	9	9	8	13	10	8	11	13	10	7	13	10	19
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3	3	1	15	3	8	3	5	7	5	9	12	10	8	9	10	6	8	10	11	9	7	13	9	13
3	3	1	16	0	8	5	3	7	9	9	11	10	8	9	10	9	12	9	10	12	9	13	10	18
3	3	1	17	0	4	9	5	4	8	9	8	8	7	8	10	9	8	11	8	11	9	13	9	11
3	3	1	18	0	4	3	4	9	8	13	7	8	8	8	7	7	9	11	13	17	12	13	10	13
3	3	1	19	2	3	3	5	4	8	9	9	8	7	6	7	8	11	10	11	11	12	6	13	7	18
3	3	1	20	1	5	7	5	6	8	6	7	7	6	7	8	11	10	11	11	12	6	13	7	18
			Ave	1.15	5.19	4.76	4.60	6.29	7.81	8.92	7.83	8.30	8.17	8.05	9.66	9.22	10.01	10.50	11.44	12.32	8.56	13.44	9.68	14.63

Group	Patient	Treat	Loc	t0 24H	t1 96H	t2 168H	t3 264H	t4 336H	t5 432H	t6 504H	t7 576H	t8 648H	t9 744H	t10 818H	t11 912H	t12 984H	t13 1080H	t14 1152H	t15 1248H	t16 1320H	t17 1416H	t18 1488H	t19 1584H	t20 1656H
3	3	2	1	4	4	3	8	4	8	7	8	9	10	11	7	12	11	11	7	8	2	6	6	7
3	3	2	2	1	9	6	4	9	7	8	7	8	8	9	6	15	10	12	10	6	4	6	3	8
3	3	2	3	2	6	8	6	9	8	10	10	10	9	12	9	14	11	9	12	9	0	8	4	9
3	3	2	4	2	2	5	5	8	10	6	10	13	15	10	10	11	8	12	9	7	2	13	3	4
3	3	2	5	0	4	3	8	6	6	11	9	9	9	10	9	12	8	11	7	12	1	9	3	7
3	3	2	6	3	3	5	6	5	11	8	10	11	11	10	9	13	11	9	9	9	0	9	11	7
3	3	2	7	0	3	3	6	5	6	8	8	10	11	15	9	14	10	11	10	7	1	7	10	9
3	3	2	8	0	3	9	6	7	12	8	12	10	7	13	11	14	11	11	8	8	0	7	3	7
3	3	2	9	3	5	5	4	5	8	6	11	12	12	12	6	12	8	8	13	6	1	10	7	4
3	3	2	10	1	6	4	5	5	7	7	7	8	9	8	6	11	10	12	10	8	1	11	3	6
3	3	2	11	5	4	4	5	4	8	14	11	9	6	11	6	12	13	9	17	6	1	9	8	7
3	3	2	12	1	2	5	10	8	13	6	10	10	9	11	7	15	10	9	12	8	0	6	3	7
3	3	2	13	1	7	4	5	5	8	8	8	8	8	13	16	14	10	8	10	8	2	9	7	11
3	3	2	14	4	2	8	4	6	9	8	14	11	7	8	9	15	9	11	10	8	0	8	4	4
3	3	2	15	1	5	5	4	6	6	9	11	10	9	12	7	16	11	11	11	8	2	12	6	6
3	3	2	16	0	4	5	6	6	8	7	9	11	13	11	12	15	7	10	8	10	0	17	8	6
3	3	2	17	1	4	3	9	5	7	11	10	10	9	11	10	16	15	17	16	8	0	9	6	7
3	3	2	18	1	4	5	5	8	8	7	13	12	11	8	8	13	10	11	7	12	1	9	4	7
3	3	2	19	0	3	5	4	6	7	8	10	8	6	11	11	12	9	11	10	8	0	6	6	6
3	3	2	20	1	4	5	6	7	8	6	10	9	8	9	11	22	9	8	8	10	1	8	6	4
			Ave	1.55	4.25	5.00	5.76	6.22	8.31	8.18	9.86	9.90	9.36	10.72	8.82	13.93	10.08	10.50	10.22	8.29	1.06	8.83	5.60	6.51

Group	Patient	Treat	Loc	t0 24H	t1 96H	t2 168H	t3 264H	t4 336H	t5 432H	t6 504H	t7 576H	t8 648H	t9 744H	t10 816H	t11 912H	t12 984H	t13 1080H	t14 1152H	t15 1248H	t16 1320H	t17 1416H	t18 1488H	t19 1584H	t20 1656H
3	3	3	1	4	4	5	9	8	11	7	4	4	4	4	7	11	7	11	6	7	5	8	6	5
3	3	3	2	2	3	3	4	6	5	10	4	5	6	4	7	7	7	8	7	8	5	8	6	6
3	3	3	3	3	4	4	4	9	7	5	4	5	6	9	7	8	8	6	9	8	3	7	7	4
3	3	3	4	5	6	4	3	8	10	7	4	6	7	15	9	9	6	7	8	6	4	8	6	5
3	3	3	5	4	3	6	5	8	7	6	10	9	7	8	8	8	8	8	8	8	6	10	7	5
3	3	3	6	2	3	4	4	11	6	5	6	7	7	6	6	8	9	9	8	11	3	8	9	6
3	3	3	7	0	3	6	5	6	8	7	6	6	6	4	7	8	7	8	8	8	3	7	7	9
3	3	3	8	2	2	3	10	7	12	6	6	7	8	8	8	10	7	7	8	12	8	7	4	7
3	3	3	9	1	3	6	6	8	9	5	7	7	6	4	6	9	7	9	11	8	10	11	7	10
3	3	3	10	0	3	4	5	4	11	8	6	8	9	16	7	8	9	7	9	8	4	7	7	4
3	3	3	11	3	6	3	6	8	5	5	8	8	7	11	7	10	9	7	7	8	3	11	6	3
3	3	3	12	3	2	4	3	8	8	7	4	6	7	8	6	10	7	9	8	7	4	6	6	5
3	3	3	13	6	3	3	5	10	12	8	9	8	6	8	6	9	7	7	8	8	10	8	7	5
3	3	3	14	1	2	2	6	8	8	7	4	6	7	8	8	12	8	8	4	6	3	6	6	7
3	3	3	15	2	3	5	4	9	8	7	7	9	10	9	6	9	7	7	7	7	6	8	6	3
3	3	3	16	2	3	7	9	6	8	13	5	6	7	8	9	10	6	10	9	11	5	8	8	3
3	3	3	17	2	2	4	6	5	5	7	6	6	6	6	7	9	6	9	8	7	6	8	8	3
3	3	3	18	2	2	5	5	13	8	9	4	7	10	4	6	9	7	8	8	6	3	13	7	3
3	3	3	19	0	2	2	3	5	8	8	5	7	9	7	7	9	9	9	8	8	3	9	8	4
			Ave	2.35	3.11	4.23	5.39	7.68	8.27	7.28	5.71	6.68	7.02	7.70	6.80	9.13	7.33	7.97	7.64	7.84	5.12	8.29	6.44	5.27

Group	Patient	Treat	Loc	t0 24H	t1 96H	t2 168H	t3 264H	t4 336H	t5 432H	t6 504H	t7 576H	t8 648H	t9 744H	t10 816H	t11 912H	t12 984H	t13 1080H	t14 1152H	t15 1248H	t16 1320H	t17 1416H	t18 1488H	t19 1584H	t20 1656H
3	4	1	1	1	6	6	9	11	13	11	13	12	11	15	16	12	14	18	21	28	11	20	21	14
3	4	1	2	2	4	6	9	11	10	11	12	12	11	12	16	16	17	15	17	21	13	17	18	17
3	4	1	3	2	6	6	12	11	9	11	13	13	12	20	12	17	11	26	13	18	12	20	21	17
3	4	1	4	0	5	5	7	9	8	9	9	10	10	16	15	18	14	17	16	21	14	12	18	12
3	4	1	5	3	5	6	7	12	11	11	11	13	15	18	16	16	13	18	12	17	16	17	18	14
3	4	1	6	2	6	6	8	8	8	12	8	12	16	13	16	20	11	17	19	21	14	26	25	14
3	4	1	7	1	9	6	11	9	13	9	4	7	10	24	18	20	15	17	13	16	14	16	21	10
3	4	1	8	1	10	6	10	9	11	11	12	12	11	13	11	11	19	16	12	19	13	17	21	16
3	4	1	9	0	6	4	8	10	12	11	12	12	11	17	26	16	13	19	13	18	12	16	26	14
3	4	1	10	1	6	4	16	11	11	10	8	9	9	17	16	15	25	13	11	20	11	15	21	11
3	4	1	11	0	6	4	7	11	11	18	12	11	10	12	16	12	12	15	15	27	15	13	25	12
3	4	1	12	3	3	7	7	11	10	14	11	11	10	17	26	16	12	17	14	21	16	17	21	17
3	4	1	13	2	5	7	8	11	8	8	18	14	9	12	11	16	14	18	11	22	16	12	19	13
3	4	1	14	3	6	8	9	14	10	13	12	11	9	16	11	15	11	17	16	20	11	18	19	15
3	4	1	15	3	6	6	9	13	12	14	9	10	11	16	13	17	13	17	14	17	14	17	16	14
3	4	1	16	4	10	9	8	12	9	8	11	11	10	17	16	16	13	15	12	27	12	13	21	12
3	4	1	17	3	6	5	9	11	12	10	11	10	9	17	15	13	12	15	12	20	17	13	18	21
3	4	1	18	1	6	4	9	9	9	11	9	10	10	17	16	16	11	13	14	21	12	19	26	14
3	4	1	19	0	6	6	9	11	8	10	13	11	9	16	21	16	14	21	13	19	16	18	20	10
3	4	1	20	1	6	8	13	14	12	15	11	11	10	16	16	18	12	17	11	26	14	17	26	16
			Ave	1.65	6.23	5.93	9.31	10.85	10.39	11.47	10.94	11.10	10.61	16.05	16.16	15.68	13.93	18.95	14.10	20.91	13.59	16.56	20.98	14.24

Group	Patient	Treat	Loc	t0 24H	t1 96H	t2 168H	t3 264H	t4 336H	t5 432H	t6 504H	t7 576H	t8 648H	t9 744H	t10 816H	t11 912H	t12 984H	t13 1080H	t14 1152H	t15 1248H	t16 1320H	t17 1416H	t18 1488H	t19 1584H	t20 1656H
3	4	2	1	4	7	4	10	12	14	11	10	12	13	15	16	20	16	15	17	27	18	22	22	18
3	4	2	2	3	5	7	10	12	18	10	13	16	19	15	22	13	19	17	22	26	17	25	18	28
3	4	2	3	1	6	7	10	12	13	13	13	12	11	17	13	15	22	17	19	21	16	22	22	22
3	4	2	4	2	11	7	10	9	10	10	13	13	13	24	13	22	19	25	27	23	18	25	24	16
3	4	2	5	0	7	6	10	12	16	12	13	14	15	16	18	13	16	20	20	25	17	24	20	17
3	4	2	6	1	5	4	10	12	14	11	13	13	13	19	16	15	27	17	15	20	18	24	21	17
3	4	2	7	1	7	9	8	14	12	10	14	13	12	17	20	16	26	13	15	18	18	25	21	21
3	4	2	8	1	7	8	11	8	17	11	15	14	13	19	12	15	17	13	19	29	17	26	18	20
3	4	2	9	2	7	6	8	8	10	10	12	12	12	19	15	10	20	13	16	17	18	24	22	20
3	4	2	10	2	9	6	9	12	18	18	18	15	11	21	11	16	19	19	20	24	16	21	26	25
3	4	2	11	1	9	7	9	12	15	15	14	14	13	15	22	12	19	17	20	22	19	25	20	18
3	4	2	12	1	9	7	9	15	14	10	14	13	11	17	13	13	19	17	21	21	25	25	29	24
3	4	2	13	4	4	7	13	11	14	10	12	12	11	17	16	16	18	17	24	23	18	21	20	24
3	4	2	14	2	11	7	13	11	11	10	13	16	18	13	16	15	19	17	20	28	18	22	18	21
3	4	2	15	4	10	6	15	12	14	13	13	13	13	17	16	15	15	21	19	21	20	28	19	20
3	4	2	16	2	7	7	10	13	11	11	12	11	10	17	17	13	17	16	24	23	15	30	31	17
3	4	2	17	0	6	7	11	13	18	12	13	13	12	17	16	15	16	15	20	21	13	22	22	20
3	4	2	18	0	4	7	10	12	12	12	12	11	10	19	12	15	16	15	16	19	20	26	24	17
3	4	2	19	0	7	9	10	14	12	11	14	13	11	17	16	15	19	17	35	23	22	30	17	21
3	4	2	20	3	9	6	10	12	14	12	15	15	15	13	12	12	19	19	17	23	18	25	20	20
			Ave	1.70	7.44	6.54	10.40	11.69	13.79	11.56	13.43	13.25	12.84	17.20	15.61	14.80	18.70	16.97	20.23	22.82	18.01	24.51	21.63	20.29

Group	Patient	Treat	Loc	t0 24H	t1 96H	t2 168H	t3 264H	t4 338H	t5 432H	t6 504H	t7 576H	t8 648H	t9 744H	t10 816H	t11 912H	t12 984H	t13 1080H	t14 1152H	t15 1248H	t16 1320H	t17 1416H	t18 1488H	t19 1584H	t20 1656H
3	4	3	1	2	2	7	7	10	14	10	14	12	9	16	15	13	17	24	11	19	22	26	12	16
3	4	3	2	3	3	7	8	11	12	9	15	16	17	12	18	19	13	18	15	18	22	27	10	22
3	4	3	3	3	3	9	8	7	9	11	16	14	11	15	17	13	18	20	17	22	18	24	13	20
3	4	3	4	3	4	5	8	7	12	11	17	15	12	17	21	20	12	18	13	20	17	26	8	19
3	4	3	5	3	5	7	11	9	9	12	17	15	12	16	17	22	26	23	11	24	22	29	12	18
3	4	3	6	5	4	7	10	13	8	8	16	14	12	16	17	12	19	36	15	21	27	34	20	25
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3	4	3	8	2	7	4	11	8	10	8	16	14	12	12	16	16	18	24	16	24	21	26	15	19
3	4	3	9	2	2	7	18	9	8	8	17	14	11	15	17	16	18	23	15	26	22	27	11	19
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3	4	3	11	0	5	6	14	10	11	13	13	12	10	17	24	11	18	20	15	22	22	28	12	17
3	4	3	12	3	2	8	7	10	14	17	16	14	12	16	17	11	16	20	19	21	22	24	9	21
3	4	3	13	1	5	10	10	7	8	11	21	17	12	16	17	16	17	26	17	21	24	46	19	19
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3	4	3	16	3	4	11	9	9	11	11	16	16	16	19	18	15	18	27	15	22	24	32	12	18
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3	4	3	19	5	4	7	12	8	11	7	15	13	10	13	19	11	28	29	22	26	25	29	12	19
3	4	3	20	2	4	4	9	12	11	11	19	14	9	21	17	15	18	19	21	19	22	29	12	18
			Ave	2.60	3.89	7.44	9.94	10.17	11.25	11.00	16.00	14.05	12.25	15.83	16.73	14.77	17.70	23.03	15.23	22.07	21.80	29.19	12.46	18.55

Group	Patient	Treat	Loc	t0 24H	t1 98H	t2 168H	t3 264H	t4 338H	t5 432H	t6 504H	t7 576H	t8 648H	t9 744H	t10 816H	t11 912H	t12 984H	t13 1080H	t14 1152H	t15 1248H	t16 1320H	t17 1416H	t18 1488H	t19 1584H	t20 1656H
3	5	1	1	1	3	2	4	4	4	10	4	5	6	6	7	6	3	12	12	7	8	6	4	12
3	5	1	2	3	1	1	4	4	4	5	7	7	6	4	9	9	6	6	8	8	8	12	8	12
3	5	1	3	3	4	3	5	3	4	5	7	7	6	6	6	6	6	9	9	6	9	8	8	13
3	5	1	4	1	2	4	2	4	3	7	9	9	8	7	6	4	7	9	11	7	9	6	6	12
3	5	1	5	0	1	1	2	4	4	7	6	6	6	9	12	4	9	9	11	8	8	6	7	12
3	5	1	6	3	3	2	4	4	5	5	6	6	6	8	8	8	12	10	13	7	8	9	9	13
3	5	1	7	2	2	3	3	5	5	8	4	5	5	6	6	6	7	6	8	8	8	6	7	12
3	5	1	8	0	2	2	4	5	5	8	5	5	5	7	10	3	3	9	7	8	6	7	7	16
3	5	1	9	0	3	2	2	5	4	7	10	7	4	6	8	6	3	6	7	6	8	8	4	15
3	5	1	10	2	1	3	3	5	5	7	4	5	6	7	9	6	7	7	10	7	8	10	6	12
3	5	1	11	2	2	1	3	3	9	7	4	6	7	6	8	13	13	13	9	7	11	6	6	8
3	5	1	13	1	1	3	3	5	4	7	7	5	3	7	6	6	4	9	9	9	8	9	7	9
3	5	1	14	2	6	3	3	5	5	7	7	5	3	6	12	7	6	9	7	7	9	7	9	11
3	5	1	15	2	2	4	5	4	3	7	9	7	4	6	13	3	3	7	11	7	7	8	6	13
3	5	1	16	3	2	3	4	3	4	6	3	5	7	6	9	6	4	6	9	7	7	9	9	13
3	5	1	17	1	2	2	2	4	5	9	6	5	3	4	6	3	6	10	7	8	8	6	10	10
3	5	1	18	1	1	1	3	3	7	6	3	4	5	6	10	4	3	10	13	9	7	7	13	13
3	5	1	19	3	1	1	7	3	3	13	6	6	5	4	6	6	6	8	9	7	8	9	4	13
3	5	1	20	1	3	2	3	4	4	6	6	6	6	6	8	6	6	7	7	8	10	9	4	13
			Ave	1.55	2.28	2.29	3.44	4.10	4.54	7.25	5.88	5.84	5.28	6.17	8.33	5.92	6.12	8.40	9.34	7.35	8.18	7.59	7.05	12.44

Group	Patient	Treat	Loc	t0 24H	t1 96H	t2 168H	t3 264H	t4 336H	t5 432H	t6 504H	t7 576H	t8 648H	t9 744H	t10 816H	t11 912H	t12 984H	t13 1080H	t14 1152H	t15 1248H	t16 1320H	t17 1416H	t18 1488H	t19 1584H	t20 1656H
3	5	2	1	1	1	3	5	5	4	2	5	5	4	6	6	4	6	6	11	9	4	3	2	7
3	5	2	2	1	1	2	5	5	3	5	5	5	4	9	4	4	7	7	6	8	3	6	6	3
3	5	2	3	3	2	3	5	3	2	8	2	3	4	7	11	3	6	9	8	6	7	4	6	4
3	5	2	4	2	2	0	3	11	4	6	6	5	4	7	4	3	7	6	8	9	5	6	3	7
3	5	2	5	2	2	4	5	6	3	7	6	6	6	6	7	4	4	8	6	8	4	3	2	6
3	5	2	6	4	2	2	7	5	2	4	4	5	5	7	6	5	4	9	6	7	5	6	4	5
3	5	2	7	3	3	4	3	5	2	2	3	5	7	7	9	5	6	8	10	7	5	6	3	3
3	5	2	8	2	2	2	6	5	2	4	5	5	5	4	4	3	7	8	6	15	9	3	4	4
3	5	2	9	1	3	0	5	7	3	3	7	5	3	7	7	4	12	10	12	7	7	5	6	5
3	5	2	10	1	6	2	4	7	3	6	6	6	6	7	8	3	8	11	8	15	6	6	2	4
3	5	2	11	1	2	1	7	3	1	6	4	5	5	7	4	6	9	8	8	9	4	7	2	7
3	5	2	12	1	3	0	5	3	4	4	3	4	4	10	7	9	4	9	7	6	6	4	4	6
3	5	2	13	1	3	1	5	4	1	4	5	6	6	10	6	3	6	11	7	8	2	4	4	5
3	5	2	14	3	1	1	6	6	2	3	7	7	7	6	7	6	9	9	8	6	2	5	6	7
3	5	2	15	0	3	0	2	3	3	3	3	3	3	9	9	8	6	9	8	9	5	3	3	3
3	5	2	16	0	1	3	5	4	3	3	5	6	7	9	11	7	6	8	9	7	4	3	3	3
3	5	2	17	4	3	0	7	8	4	4	5	4	3	6	6	6	4	8	8	15	4	8	4	5
3	5	2	18	3	3	0	8	5	4	5	6	6	5	4	4	9	6	6	8	7	4	11	6	6
3	5	2	19	0	5	2	4	3	3	2	5	6	6	8	7	3	4	8	8	15	2	4	3	6
3	5	2	20	2	4	2	3	5	6	2	5	6	8	6	4	3	6	11	8	7	3	6	2	5
			Ave	1.75	2.53	1.60	5.00	5.18	2.94	4.17	4.60	5.15	5.18	7.02	6.51	5.04	6.50	8.43	7.84	8.62	4.62	5.23	3.86	5.08

Group	Patient	Treat	Loc	t0 24H	t1 96H	t2 168H	t3 264H	t4 336H	t5 432H	t6 504H	t7 576H	t8 648H	t9 744H	t10 816H	t11 912H	t12 984H	t13 1080H	t14 1152H	t15 1248H	t16 1320H	t17 1416H	t18 1488H	t19 1584H	t20 1656H
3	5	3	1	2	2	3	5	2	4	5	3	5	7	6	7	8	7	11	7	7	3	4	2	9
3	5	3	2	5	1	2	5	3	3	3	7	7	7	6	7	4	6	10	9	5	3	4	4	3
3	5	3	3	3	2	1	4	9	4	4	9	6	3	7	7	7	6	8	11	11	3	4	2	2
3	5	3	4	4	1	3	4	2	5	3	4	6	7	6	7	5	10	6	6	6	1	2	6	4
3	5	3	5	0	1	5	8	2	7	2	5	6	7	4	7	6	6	10	8	7	3	4	7	3
3	5	3	6	1	3	1	5	2	4	3	7	7	7	7	13	3	4	7	10	5	2	3	3	3
3	5	3	7	4	2	1	5	1	3	6	7	8	8	6	4	7	8	7	7	7	2	4	4	4
3	5	3	8	0	4	2	6	3	3	4	5	4	3	4	7	5	8	7	8	5	3	4	4	2
3	5	3	9	2	2	5	4	4	7	6	3	5	7	6	7	3	7	12	10	6	4	6	6	4
3	5	3	10	2	2	2	7	2	6	2	8	9	10	8	6	6	8	8	8	8	2	3	2	4
3	5	3	11	2	2	1	5	3	4	4	3	4	4	8	9	6	7	11	7	7	3	2	4	2
3	5	3	12	1	2	2	5	5	5	3	4	5	6	6	10	7	11	7	10	7	3	4	7	6
3	5	3	13	3	1	1	4	1	9	3	3	5	6	6	7	5	4	13	6	7	1	6	4	7
3	5	3	14	6	1	2	7	2	9	5	5	8	10	10	7	4	4	12	9	7	6	4	4	4
3	5	3	15	1	2	5	5	3	5	4	5	5	4	6	9	6	7	17	10	6	4	2	4	8
3	5	3	16	3	3	1	4	1	6	11	4	6	8	6	7	6	12	10	9	8	7	6	3	6
3	5	3	17	0	3	5	4	3	5	2	4	5	6	6	7	3	7	8	6	6	3	4	4	2
3	5	3	18	1	1	2	4	1	6	4	5	6	7	6	4	4	7	8	7	6	1	4	2	4
3	5	3	19	1	2	2	7	3	3	2	5	9	12	9	10	4	4	10	8	7	3	7	3	3
3	5	3	20	2	2	2	5	1	8	8	5	6	6	4	4	4	7	11	8	9	2	8	3	4
			Ave	2.15	1.94	2.47	5.20	2.63	5.35	4.21	5.06	6.10	6.65	6.35	7.39	5.20	7.00	9.68	8.12	6.74	3.16	4.48	4.08	4.41

Addendum D: Percentage increase in cell numbers and growth curves of individual cell lines.

Table D.1. Percentage increase in cell numbers over a period of ten weeks.

	T0 24H	T1 96H	T2 168H	T3 264H	T4 336H	T5 432H	T6 504H	T7 576H	T8 648H	T9 744H	T10 816H	T11 912H	T12 984H	T13 1080H	T14 1152H	T15 1248H	T16 1320H	T17 1416H	T18 1488H	T19 1584H	T20 1656H
K1MEM	2.10	2.47	2.53	2.19	1.45	3.00	5.06	5.39	5.94	5.13	5.38	6.94	8.00	5.07	4.71	9.00	7.93	5.33	5.13	8.40	7.76
K1CYC	2.90	5.25	4.11	4.41	2.79	5.12	5.00	7.18	5.40	7.14	7.28	8.89	8.51	6.56	7.49	10.22	11.53	6.50	6.23	8.08	10.01
K1CA	2.60	4.61	5.06	5.94	5.94	3.78	6.33	8.13	6.18	5.50	7.80	7.39	9.29	5.89	4.94	8.33	8.73	7.00	5.29	5.44	7.89
K2MEM	0.95	2.59	7.29	5.13	5.27	5.05	6.63	10.93	10.88	9.69	9.12	11.81	22.00	9.25	9.31	12.80	14.07	15.81	13.08	13.06	7.89
K2CYC	2.40	2.26	3.41	5.87	5.12	8.20	5.33	4.69	10.06	6.47	9.00	6.47	8.27	6.73	10.59	10.43	7.06	6.76	6.13	9.28	7.67
K2CA	2.90	3.25	9.47	5.76	5.59	9.06	8.07	10.38	10.06	10.67	12.93	14.00	15.40	11.71	9.19	13.56	12.50	11.12	7.94	11.27	13.07
K3MEM	2.15	4.87	5.88	12.76	5.65	6.20	13.27	13.57	21.71	23.21	20.93	14.88	21.13	20.82	18.79	27.23	29.47	19.19	21.13	16.21	27.67
K3CYC	3.15	6.61	8.80	13.47	11.67	14.47	22.93	26.00	29.93	32.29	32.87	47.86	45.13	25.80	25.93	39.13	35.27	37.40	33.40	36.13	23.21
K3CA	1.50	5.00	7.08	15.57	10.88	12.94	14.93	15.00	20.13	26.07	26.53	22.67	32.21	28.86	22.00	48.69	26.43	24.00	24.89	32.36	27.33
K4MEM	1.25	2.94	4.24	5.33	3.75	4.06	9.64	8.67	10.17	18.00	11.87	16.07	12.60	22.00	12.29	18.07	13.13	18.47	22.41	16.94	18.33
K4CYC	2.30	2.39	3.41	8.94	5.73	7.00	10.56	13.71	15.18	15.93	16.85	13.36	24.79	20.36	14.35	23.93	16.50	24.54	18.63	16.53	31.73
K4CA	2.00	2.65	4.80	6.00	5.18	7.53	9.35	8.94	17.73	16.06	9.24	15.36	17.27	20.00	14.40	18.92	16.67	14.05	14.71	13.19	16.56
K5MEM	0.75	4.47	7.06	4.81	5.00	4.81	6.78	7.24	6.79	10.40	10.18	21.31	11.87	9.11	9.71	12.87	12.06	11.00	13.07	16.27	12.93
K5CYC	2.35	5.73	8.39	12.40	5.12	9.53	9.40	7.88	7.29	11.13	12.73	14.93	19.53	14.13	13.27	16.69	11.50	16.73	13.06	16.06	10.93
K5CA	3.70	3.44	5.88	9.75	7.88	9.12	8.94	10.00	8.87	10.59	9.88	14.14	16.71	11.73	12.88	16.79	19.00	14.19	14.47	15.62	18.07
R1MEM	0.80	4.23	6.15	9.89	6.76	8.41	11.76	12.33	15.31	18.19	16.79	20.06	22.36	19.20	15.96	24.70	20.00	19.95	19.53	20.37	20.75
R1CYC	0.80	1.65	3.76	4.14	5.50	8.38	6.25	6.71	7.36	8.01	5.30	6.46	6.89	5.34	7.99	10.30	7.84	5.82	4.22	4.28	0.56
R1CA	0.65	1.88	2.25	2.50	4.24	5.72	3.68	3.68	5.01	6.32	3.92	5.52	5.84	3.71	8.44	9.52	5.88	7.70	2.10	6.08	5.15
R2MEM	1.45	5.50	6.29	9.53	9.75	15.36	12.13	12.81	12.53	12.25	16.00	18.44	20.93	16.80	26.32	29.21	25.42	15.31	11.41	18.14	14.70
R2CYC	1.20	5.53	8.43	7.39	10.36	11.43	12.07	16.33	15.56	14.78	18.74	21.06	17.57	24.72	25.83	27.07	27.20	15.23	7.41	14.48	7.91
R2CA	0.80	3.57	4.82	6.39	12.07	12.38	11.00	15.12	14.40	13.68	20.80	20.62	13.09	23.28	34.48	32.41	42.19	19.12	8.75	20.63	8.59
R3MEM	3.10	6.30	7.10	7.35	10.80	8.80	8.40	6.45	8.35	10.25	13.80	11.95	8.80	9.35	9.90	13.80	15.35	9.40	8.90	8.50	7.90
R3CYC	1.00	6.36	6.00	8.29	8.79	8.69	9.33	8.50	8.77	9.03	8.36	10.02	6.93	6.98	12.12	15.44	12.45	2.59	5.60	9.29	5.92
R3CA	2.00	3.45	4.28	8.88	7.87	9.56	10.38	11.00	10.47	9.94	10.67	12.95	7.84	8.17	17.83	17.68	16.65	3.61	7.28	8.03	6.48
R4MEM	2.10	3.00	4.71	4.47	3.44	1.50	5.00	8.20	7.53	8.50	8.75	12.07	4.89	7.56	9.14	7.71	5.50	7.13	5.67	5.80	9.33
R4CYC	1.65	5.44	3.47	2.59	5.31	1.00	6.80	8.93	7.56	8.63	13.93	6.82	6.25	9.56	9.69	8.76	6.13	6.79	6.29	7.29	5.40
R4CA	2.70	7.53	4.33	3.56	5.69	1.30	5.22	9.25	7.50	10.06	9.33	10.50	7.63	7.53	10.38	13.69	7.25	8.71	7.50	8.33	7.00
R5MEM	1.80	5.73	7.11	8.19	10.36	14.94	11.24	12.57	13.57	14.56	14.82	18.56	17.60	16.11	26.57	23.71	20.72	18.38	26.24	21.76	17.85
R5CYC	2.20	6.80	8.13	11.87	14.40	16.44	14.25	17.75	17.28	16.80	20.48	20.96	17.92	16.73	20.23	22.05	15.23	8.96	15.82	10.20	8.06
R5CA	1.35	3.67	6.71	8.68	9.58	12.47	10.44	16.07	15.35	14.00	15.28	22.03	18.97	16.50	21.50	20.37	19.86	8.96	14.02	13.36	16.24
NR1MEM	1.20	3.53	3.67	5.19	6.65	6.67	6.11	5.44	6.57	7.67	8.88	8.63	10.01	10.08	9.21	10.21	6.22	9.26	11.52	8.08	9.58
NR1CYC	1.75	4.00	4.89	5.11	7.13	9.93	5.84	7.20	6.96	6.72	6.52	7.32	7.96	9.49	9.37	12.69	8.21	5.92	10.14	4.76	1.18
NR1CA	1.40	3.56	4.40	4.60	7.13	7.94	6.93	4.75	5.88	7.00	6.65	8.68	9.38	9.15	13.07	13.02	8.44	4.90	4.91	3.86	4.76
NR2MEM	1.00	2.37	0.95	0.15	0.95	2.41	1.35	2.00	2.58	5.75	5.61	6.41	4.28	3.69	6.73	5.38	4.75	5.71	9.79	8.40	4.81
NR2CYC	2.30	3.73	2.63	2.53	3.78	4.73	5.47	5.00	4.76	7.73	7.57	12.80	12.85	4.00	7.29	8.00	8.60	7.24	8.13	9.17	6.35
NR2CA	3.15	3.89	4.53	2.33	3.47	4.19	5.82	3.88	3.88	9.31	8.61	8.86	8.93	5.24	7.67	6.12	5.50	5.39	9.47	8.53	6.76
NR3MEM	1.15	5.19	4.76	4.60	6.29	7.81	8.92	7.83	8.00	8.17	8.05	9.66	9.22	10.01	10.50	11.44	12.32	8.56	13.44	9.68	14.63
NR3CYC	1.55	4.25	5.00	5.76	6.22	8.31	8.16	9.86	9.61	9.36	10.72	8.82	13.93	10.08	10.50	10.22	8.29	1.06	8.63	5.60	6.51
NR3CA	2.35	3.06	4.17	5.39	7.80	8.27	7.31	5.72	6.42	7.12	7.70	6.79	9.29	7.24	7.91	7.65	7.84	5.20	8.21	6.51	5.23
NR4MEM	1.65	6.23	5.93	9.31	10.85	10.39	11.47	10.94	10.78	10.61	16.05	16.16	15.68	13.93	16.95	14.10	20.91	13.59	16.56	20.98	14.24
NR4CYC	1.70	7.44	6.54	10.40	11.69	13.79	11.56	13.43	13.14	12.84	17.20	15.61	14.80	18.70	16.97	20.23	22.62	18.01	24.51	21.63	20.29
NR4CA	2.60	3.89	7.44	9.94	10.17	11.25	11.00	16.00	14.13	12.25	15.63	16.73	14.77	17.70	23.03	15.23	22.07	21.80	29.19	12.46	18.55
NR5MEM	1.55	2.29	2.28	3.67	4.29	4.67	7.13	5.73	5.46	5.18	6.25	8.33	5.92	6.12	8.65	9.21	7.32	8.27	7.59	7.05	12.32
NR5CYC	1.75	2.53	1.60	5.00	5.18	2.94	4.17	4.80	4.99	5.18	7.02	6.51	5.04	6.50	8.43	7.84	8.82	4.62	5.23	3.86	5.08
NR5CA	2.15	1.94	2.47	5.20	2.63	5.35	4.21	5.06	5.86	6.65	6.35	7.39	5.20	7.00	9.68	8.12	6.74	3.16	4.48	4.08	4.41

Addendum D: Percentage increase in cell numbers and growth curves of individual cell lines.

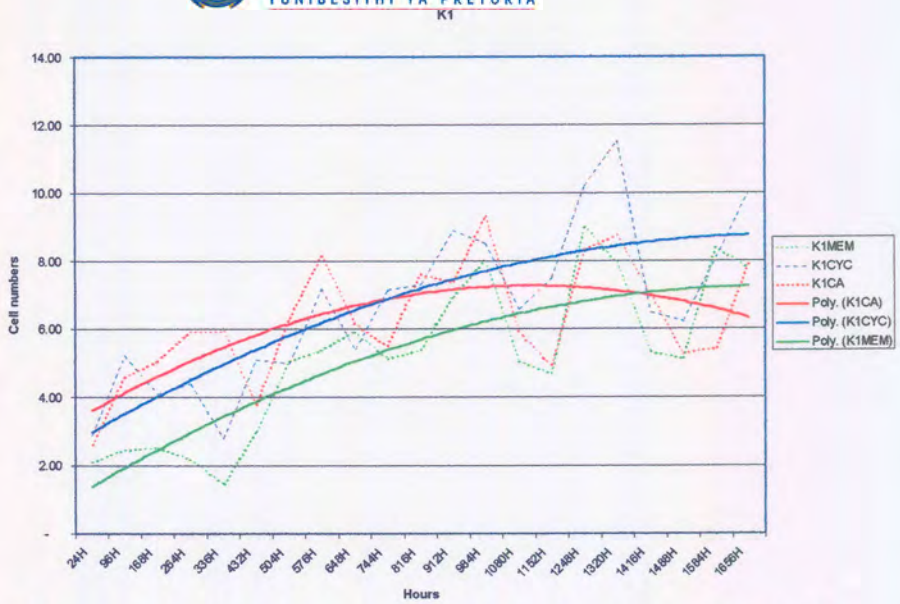

Figure D.1: Growth curve of individual cell line - Control 1

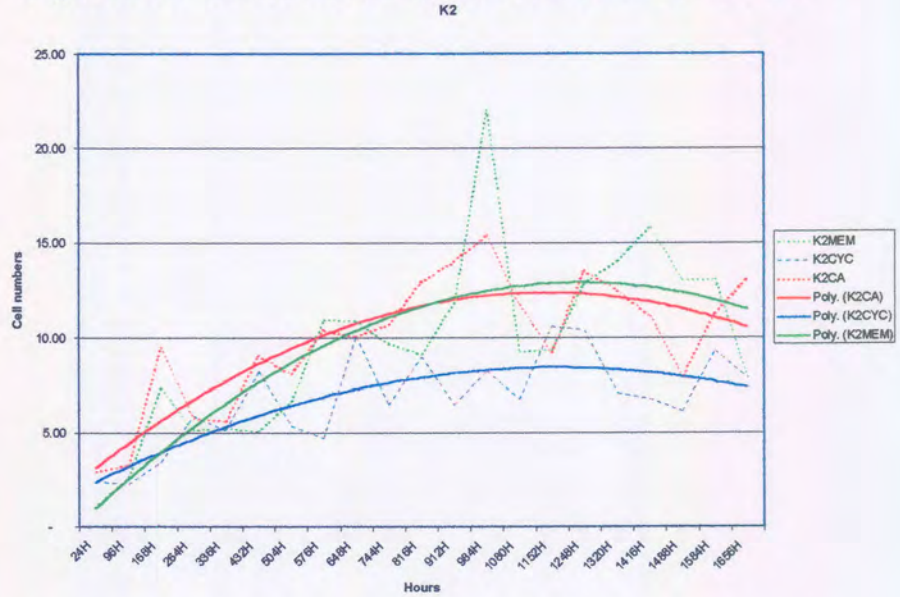

Figure D.2: Growth curve of individual cell line - Control 2

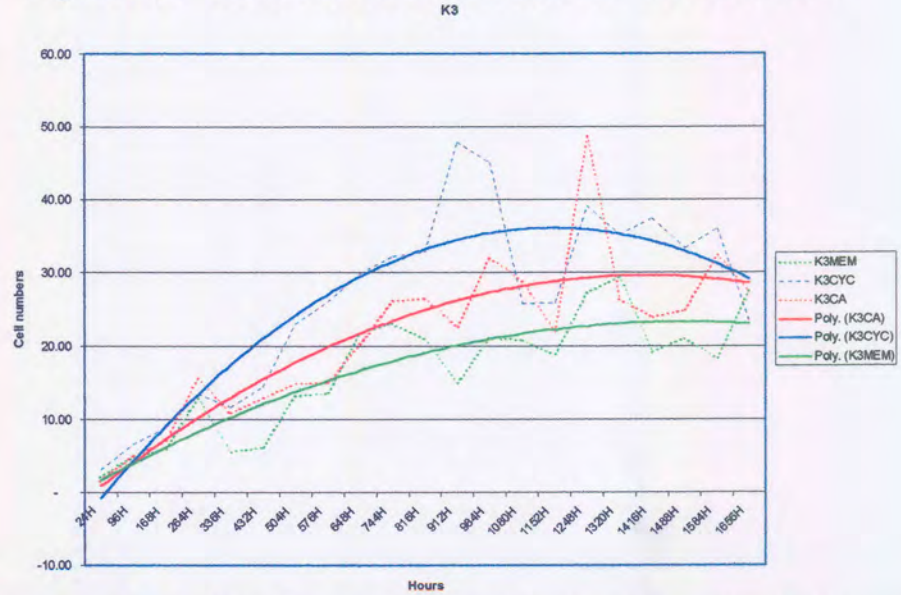

Figure D.3: Growth curve of individual cell line - Control 3

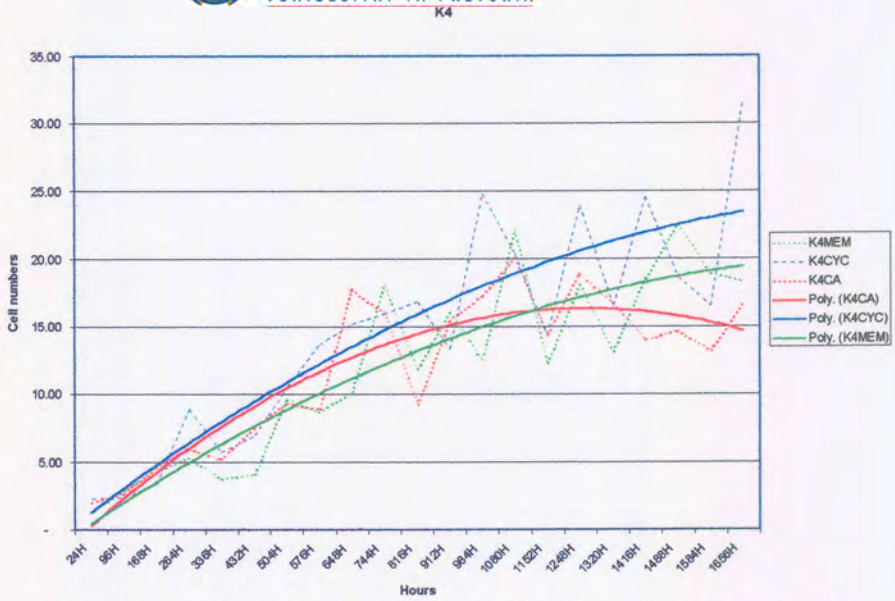

Figure D.4: Growth curve of individual cell line - Control 4

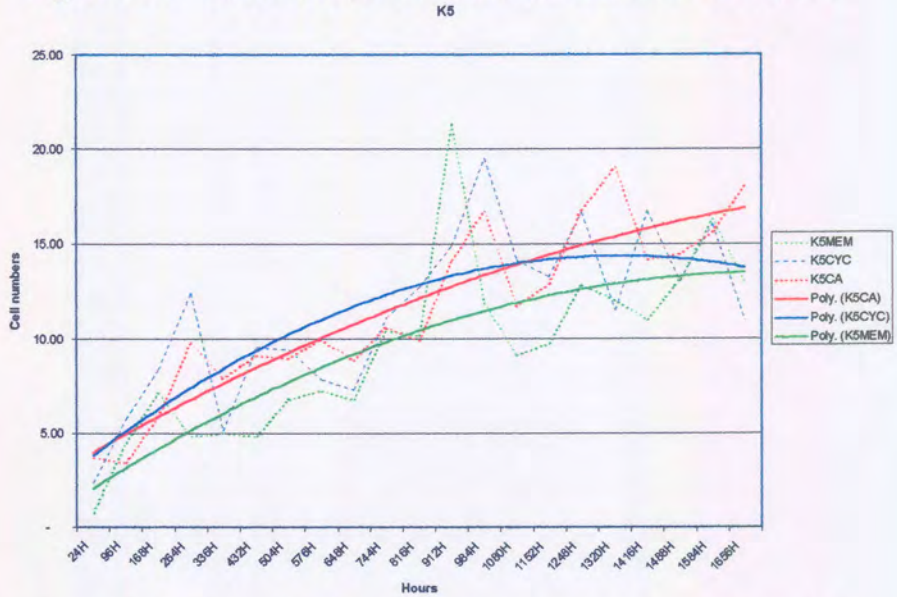

Figure D.5: Growth curve of individual cell line - Control 5

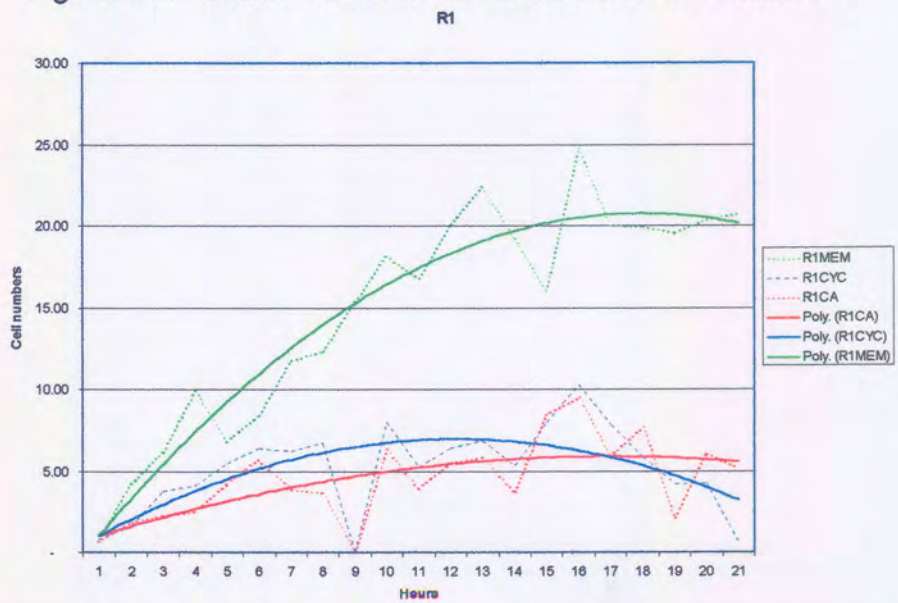

Figure D.6: Growth curve of individual cell line - Responder 1

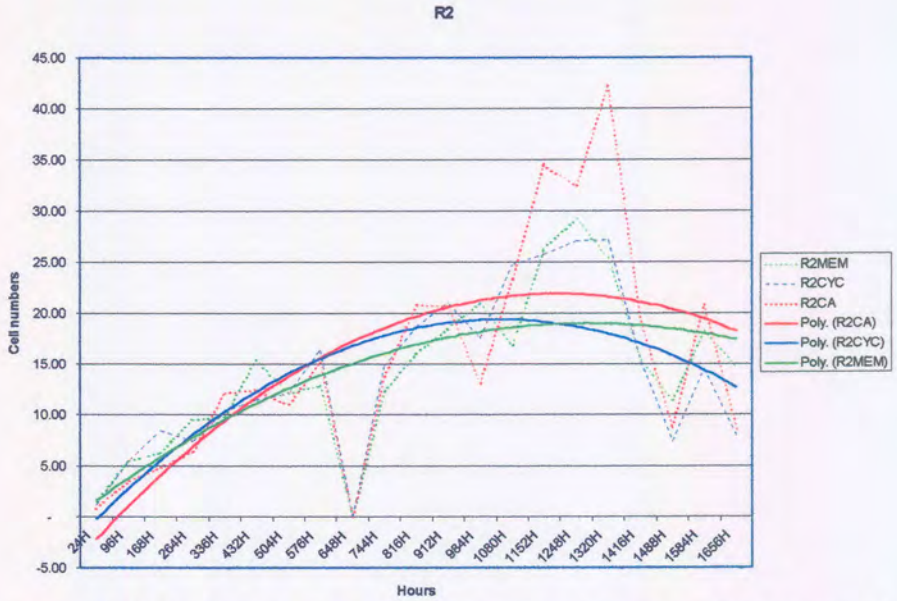

Figure D.7: Growth curve of individual cell line - Responder 2

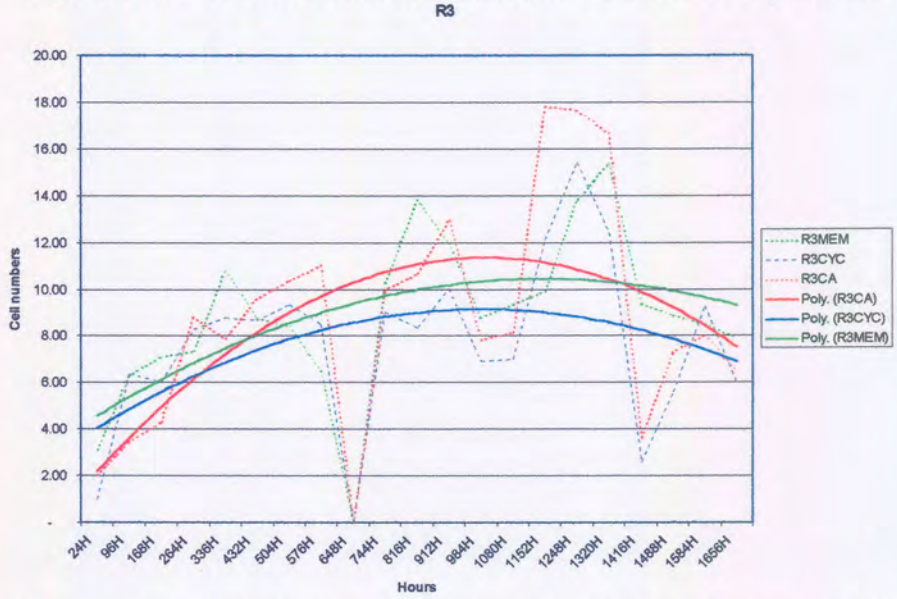

Figure D.8: Growth curve of individual cell line - Responder 3

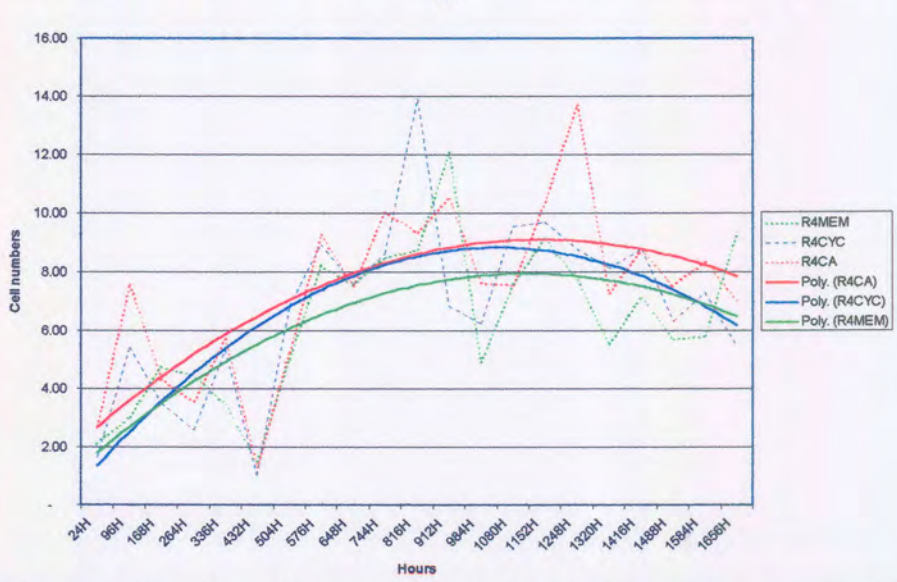

Figure D.9: Growth curve of individual cell line - Responder 4

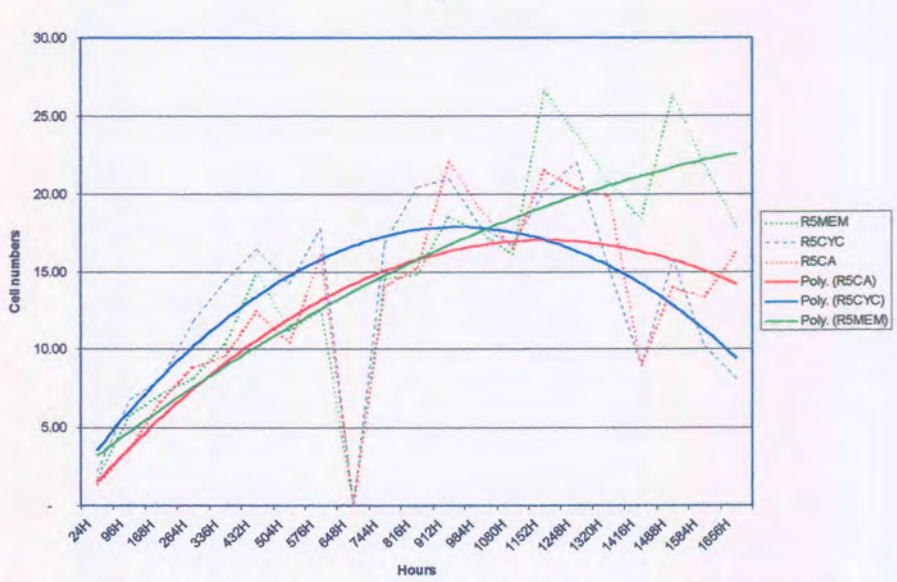

Figure D.10: Growth curve of individual cell line - Responder 5

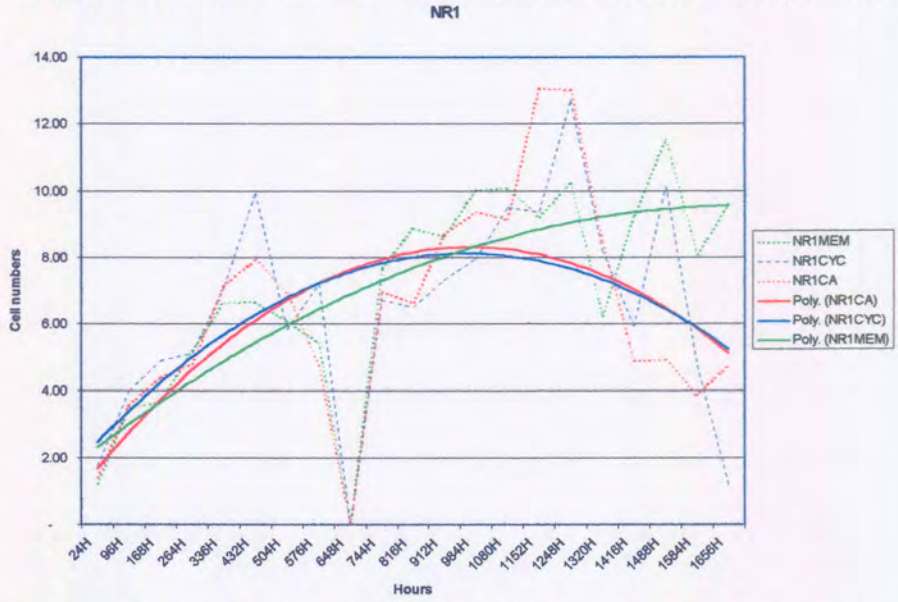

Figure D.11: Growth curve of individual cell line - Non-responder 1

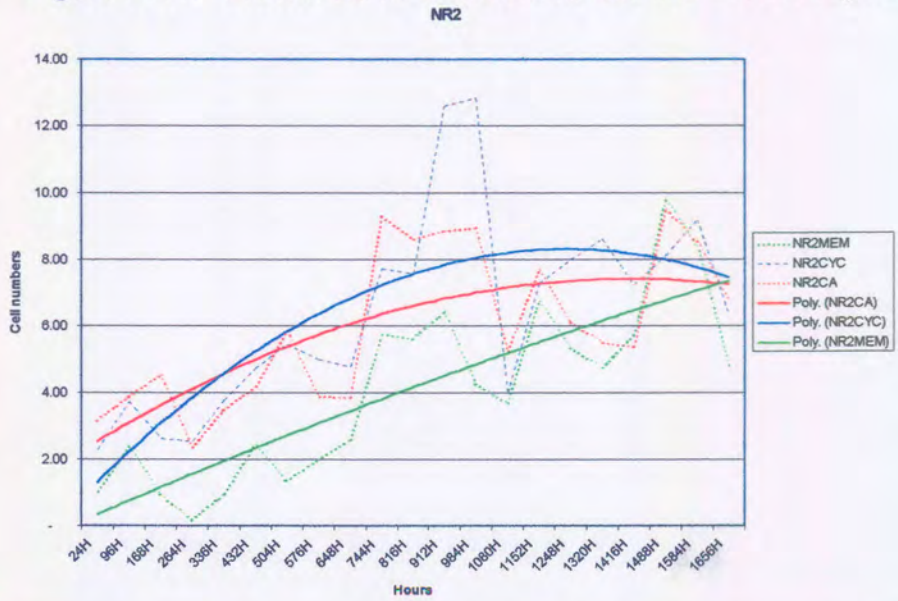

Figure D.12: Growth curve of individual cell line - Non-responder 2

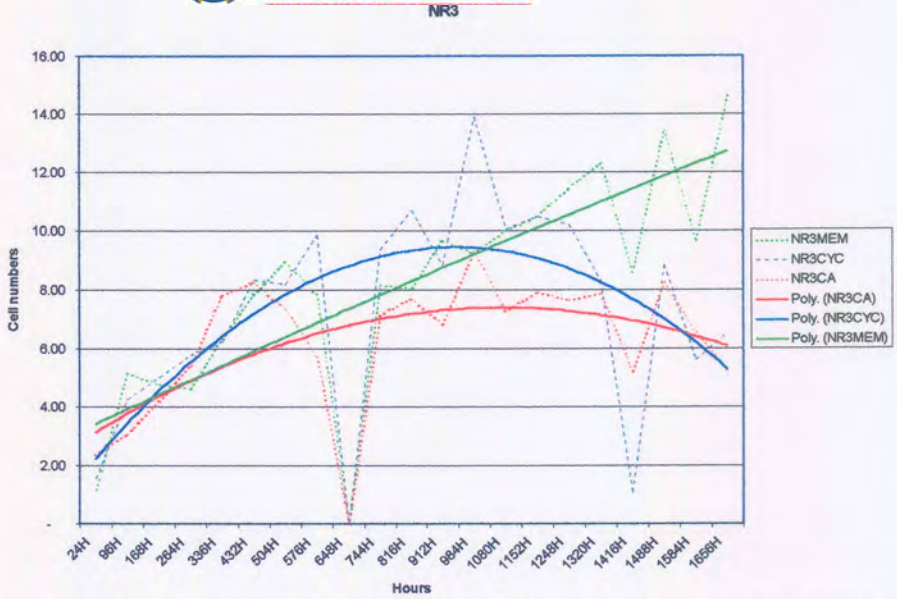

Figure D.13: Growth curve of individual cell line - Non-responder 3

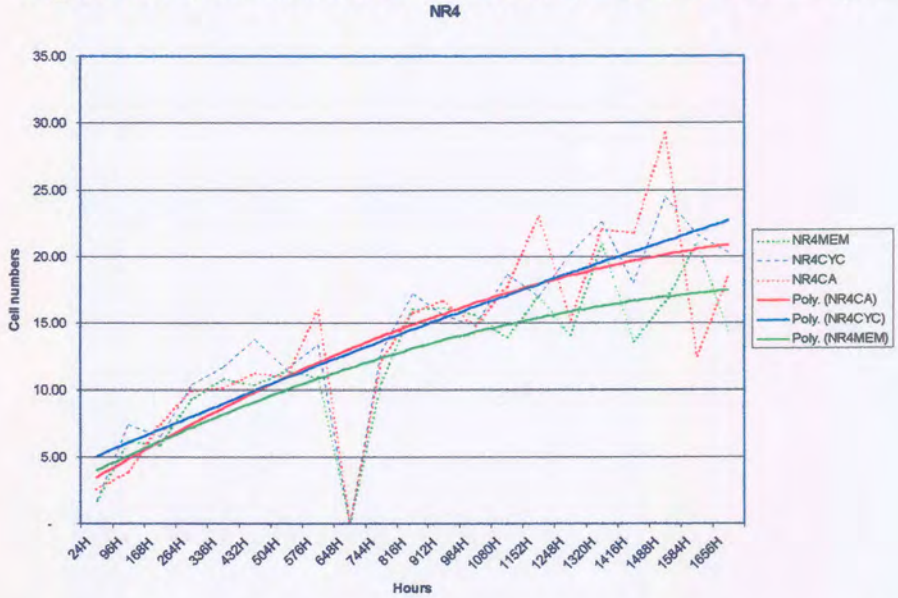

Figure D.14: Growth curve of individual cell line - Non-responder 4

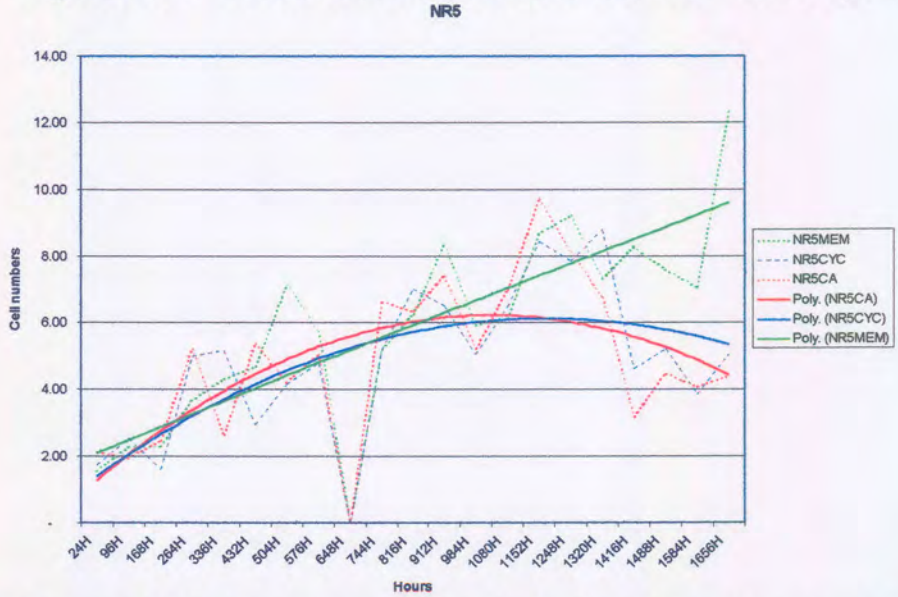

Figure D.15: Growth curve of individual cell line - Non-responder 5

Addendum E: Results obtained by the Flexible Image Processing System (FIPS).

Control K1
EMEM
Collagen type VI

Number	Area	Perimeter
1	0.31	6.88
2	6.87	42.87
3	0.56	6.11
4	0.48	6.52
5	3.75	11.63
6	0.24	3.76
7	0.12	3
8	0.11	2.67
9	0.3	5.98
10	0.31	4.98
11	0.44	7.43
12	0.88	7.88
13	1.51	14.76
14	1.25	11.76
15	0.18	13.37

Control K1
CYC
Collagen type VI

Number	Area	Perimeter
1	5	32.74
2	2.04	18.93
3	1.94	14.6
4	0.13	1.61
5	0.55	8.32
6	0.17	1.78
7	0.88	8.82
8	0.17	2.27
9	0.53	5.25
10	0.34	4.48
11	0.2	2.07
12	0.18	1.88

Control K1
CA
Collagen type VI

Number	Area	Perimeter
1	0.84	7.82
2	0.2	1.87
3	0.19	2.05
4	0.51	5.52
5	0.65	4.65
6	0.19	1.95
7	1.49	8.52
8	2.45	27.34
9	1.55	13.15
10	0.48	4.46
11	0.16	2.48
12	1.92	17.2
13	5.25	37
14	0.35	4.08
15	0.67	5.28
16	1.69	10.27
17	0.15	1.6
18	0.82	7.19
19	1.82	11.17
20	4.46	4.46

Number of objects	15	12	20
Block size	337.309 sqr mm	342.712 sqr mm	337.309sqr mm
Average Perimeter	9.973 mm	8.564 mm	8.903mm
Average size	1.153 sqr mm	1.011 sqr mm	1.095 sqr mm
Average Feret	1.892mm	1.575 mm	1.729mm
Density of objects	0.05101/sqr mm	0.035 sqr / mm	0.05929 / sqr mm
Total area size	17.302 sqr mm	12.129 sqr mm	21.905 Sqr mm
Percentage cover	5.13%	3.54%	6.49%

Non-responder R1
CYC
Collagen type IV

Number	Area	Perimeter
1	0.42	3.85
2	0.4	5.68
3	0.44	5.73
4	1.01	8
5	4.32	30.4

Non-responder R1
CYC
Collagen type V

Number	Area	Perimeter
1	0.67	7.19
2	0.22	1.96
3	0.39	3.42
4	0.21	2.4
5	0.28	4.92
6	0.64	5.13
7	0.19	2.33

Non-responder R1
EMEM
Collagen type VI

Number	Area	Perimeter
1	0.57	9.25
2	0.21	2.96
3	0.44	7.32
4	2	18.86
5	0.41	3.2
6	0.21	2.77
7	0.2	3.56
8	0.52	6.49
9	0.54	4.02
10	2.09	17.61
11	0.45	5.63
12	0.26	4.28
13	0.27	3.04
14	0.28	2.57

Number of objects	14	5	7
Block size	337.667 sqr mm	343.975 sqr mm	338.745 sqr mm
Average Perimeter	6.539 mm	10.733	3.908 mm
Average size	0.597 sqr mm	1.318 sqr mm	0.372 sqr mm
Average Feret	1.617 mm	1.999 mm	0.980 mm
Density of objects	0.04143 / sqr mm	0.01454 / sqr mm	0.02066 / sqr mm
Total area size	8.356 sqr mm	6.592 sqr mm	2.607 sqr mm
Percentage cover	2.48%	1.92%	0.77%

Non -responder R1
CYC
Collagen type VI

Number	Area	Perimeter
1	7.44	33.73
2	11.88	47.5
3	1.91	15.14
4	2.26	15.83
5	2.72	22.42
6	0.59	7.17
7	0.61	8.39

Non-responder R1
CA
Collagen type V

Number	Area	Perimeter
1	0.88	5.4
2	0.23	2.16
3	0.78	4.12
4	0.37	3.2
5	0.06	1.05

Non-responder R1
CA
Collagen type VI

Number	Area	Perimeter
1	0.07	1.31
2	0.07	0.97
3	0.38	5.06
4	0.72	9.16
5	2.96	21.55
6	0.18	2.54
7	1.37	10.39
8	0.53	5.49
9	0.79	6.43
10	1.14	15.56
11	1.65	12.7
12	0.33	3.67
13	0.96	10.44
14	0.09	1.43
15	0.24	3.81
16	0.1	1.97
17	0.31	4.49

Number of objects	7	5	17
Block size	339.103 sqr mm	337.667 sqr mm	338.385 sqr mm
Average Perimeter	21.455	3.185 mm	6.879 mm
Average size	3.916 sqr mm	0.463 sqr mm	0.7 sqr mm
Average Feret	3.341 mm	0.907 mm	1.377
Density of objects	0.02064 / sqr mm	0.01481 / sqr mm	0.05024 / sqr mm
Total area size	27.411 sqr mm	2.316 sqr mm	11.908 sqr mm
Percentage cover	8.08%	0.69%	3.52%

Non-responder R2
EMEM
Collagen type VI

Number	Area	Perimeter
1	0.23	2.84
2	0.66	7.31
3	0.5	4.22
4	0.29	2.38
5	0.26	2.7
6	0.52	5.75
7	1.22	17.07
8	4.56	39.95
9	1.73	13.6
10	0.28	2.74

Non-responder R2
CYC
Collagen type VI

Number	Area	Perimeter
1	0.97	8.48
2	0.07	1.12
3	0.12	1.52
4	1.64	8.8
5	0.5	5.6
6	0.96	10.58
7	3.46	20.01
8	8.18	59.66
9	0.23	2.01
10	0.79	5.87
11	0.8	8.84
12	0.71	7.02
13	1.34	8.62
14	0.87	9.45
15	7.26	38.2
16	1.24	9.36
17	0.54	3.82
18	0.66	8.2
19	0.18	1.87
20	2.05	16.71
21	0.99	9.04
22	2.99	20.33
23	0.51	5.05

Non-responder R2
CA
Collagen type V

Number	Area	Perimeter
1	0.33	3.49
2	0.1	1.87
3	0.54	4.31
4	0.49	3.33

Number of objects	10	23	4
Block size	337.847 sqr mm	339.103	338.745 sqr mm
Average Perimeter	9.856 mm	11.747 mm	3.251 mm
Average size	1.025 sqr mm	1.610 sqr mm	0.366 sqr mm
Average Feret	1.915 mm	2.168 mm	0.893 mm
Density of objects	0.0296 / sqr mm	0.06783 / sqr mm	0.01181 / sqr mm
Total area size	10.253 sqr mm	37.036 sqr mm	1.465 sqr mm
Percentage cover	3.04%	10.92%	0.43%

Non-responder R2
CA
Collagen type VI

Number	Area	Perimeter
1	0.52	6.1
2	0.42	3.66
3	1.31	13.87
4	0.43	3.54
5	0.19	3.02
6	1.76	13.32
7	0.72	6.79
8	0.36	7.01
9	0.81	8.86
10	0.44	4.87
11	0.75	8.11
12	0.4	7.23
13	0.25	4.53
14	0.18	2.39
15	0.55	6.44
16	0.64	5.47

Non-responder R3
EMEM
Collagen type VI

Number	Area	Perimeter
1	22	3.07
2	0.06	1.05
3	1.04	11.3
4	0.08	1.17
5	0.18	2.54
6	0.75	9.22
7	0.44	4.03
8	0.12	2.33
9	0.19	2.56
10	0.2	2.54

Non-responder R3
CYC
Collagen type IV

Number	Area	Perimeter
1	0.21	2.93
2	0.29	4.93
3	0.78	6.72
4	0.08	1.17
5	0.32	2.8

Number of objects	16	10	5
Block size	338.745 sqr mm	338.385 sqr mm	339.285 sqr mm
Average Perimeter	6.575 mm	3.980 mm	3.708 mm
Average size	0.608 sqr mm	0.329 sqr mm	0.336 sqr mm
Average Feret	1.568	0.955 mm	0.989 mm
Density of objects	0.04723 / sqr mm	0.02955 / sqr mm	0.01474 / sqr mm
Total area size	9.728 sqr mm	3.289 sqr mm	1.68
Percentage cover	2.87%	0.97%	0.50%

Non-responder R3
CYC
Collagen typeVI

Number	Area	Perimeter
1	0.5	8.78
2	0.15	2.06
3	0.16	2.11
4	0.35	4.99
5	0.7	8.62
6	1.19	6.94
7	3.81	21.15
8	1.62	9.34
9	0.54	6.16
10	0.41	3.26
11	0.85	12.1
12	0.45	6.79
13	5.21	25.65
14	0.21	3.67
15	2.74	14.27
16	5.39	16.44
17	0.09	1.64
18	0.3	4.25

Non-responder R3
CA
Collagen type V

Number	Area	Perimeter
1	0.11	1.45
2	0.09	1.22
3	0.09	1.36
4	0.4	4.26

Non-responder R3
CA
Collagen type VI

Number	Area	Perimeter
1	2.52	28.78
2	0.11	2.06
3	1.2	15.7
4	0.32	4.24
5	0.17	3.89
6	0.93	10.25
7	0.43	7.62
8	1.01	16.03
9	0.29	3.99
10	1.68	25.14
11	1.1	10.68
12	0.16	3.92
13	0.26	4.35
14	0.72	10.5
15	0.08	1.23
16	0.18	1.91
17	0.3	4.93
18	0.27	3.63

Number of objects	18	4	18
Block size	337.847 sqr mm	338.745 sqr mm	337.847 sqr mm
Average Perimeter	8.790 mm	2.071 mm	8.825 mm
Average size	1.370 sqr mm	0.173 sqr mm	0.652 sqr mm
Average Feret	2.002 mm	0.592 mm	0.435 mm
Density of objects	0.05328 /sqr mm	0.01181 / sqr mm	0.07992 / sqr mm
Total area size	24.661 sqr mm	0.692 sqr mm	11.732 sqr mm
Percentage cover	7.30%	0.20%	3.47%

Non-responder R4
EMEM
Collagen type VI

Number	Area	Perimeter
1	0.21	3.03
2	0.25	3.29
3	1.03	10.54
4	0.65	10.09
5	0.19	2.25
6	0.15	2.4
7	0.27	3.69
8	0.77	5.82
9	0.8	11.88
10	0.61	6.39
11	0.11	2.18
12	0.08	1.84
13	0.42	5.94
14	0.24	3.44
15	0.39	7.38
16	0.27	3.22
17	0.23	2.68
18	0.65	6.5
19	0.49	5.82
20	0.17	1.87

Non-responder R4
CYC
Collagen type IV

Number	Area	Perimeter
1	0.48	3.04
2	0.17	1.97

Non-responder R4
CYC
Collagen type VI

Number	Area	Perimeter
1	1.08	9.71
2	1.22	19.57
3	2.25	15.02
4	0.68	8.04
5	0.77	8.97
6	0.72	7.02
7	0.2	2.83
8	2.88	23.85

Number of objects	20	2	8
Block size	337.667 sqr mm	336.771 sqr mm	338.562 sqr mm
Average Perimeter	5.013 mm	2.505 mm	11.875 mm
Average size	0.399 sqr mm	0.323 sqr mm	1.225 sqr mm
Average Feret	1.122 mm	.750 mm	2.069 mm
Density of objects	0.05927 sqr mm	0.00594 sqr mm	0.02363 sqr mm
Total area size	7.979 sqr mm	0.645 sqr mm	9.804 sqr mm
Percentage cover	2.36%	0.19%	2.89%

Non-responder R4
CA
Collagen type VI

Number	Area	Perimeter
1	0.77	5.54
2	0.8	5.34
3	0.61	5.94
4	2.43	16.48
5	1.29	11.02
6	0.95	5.17
7	0.24	2.23
8	0.68	6.19
9	2.15	22.63
10	0.38	3.65
11	0.19	1.84
12	2.25	20.36
13	0.05	0.89
14	2.84	15.1
15	0.47	3.02
16	0.52	6.32
17	0.29	2.97
18	0.88	6.33
19	2.18	15.37
20	4.56	32.05

Non-responder R5
EMEM
Collagen type VI

Number	Area	Perimeter
1	0.36	6.84
2	0.28	3
3	0.22	3.23
4	0.35	2.75
5	1.49	15.12
6	0.09	1.54
7	0.32	3.1
8	0.55	5.44
9	1.92	13.75
10	0.8	7.89
11	0.36	3.11
12	0.13	2.51
13	0.22	3
14	0.35	3.63
15	1.85	13.43
16	0.36	3.94
17	1.47	8.92
18	0.57	7.93

Non-responder R5
CYC
Collagen type VI

Number	Area	Perimeter
1	3.18	30.1
2	1.23	12.43
3	0.73	7.3
4	0.31	4.03
5	0.74	8.89
6	0.51	6.06
7	0.12	1.97
8	0.74	9.1
9	0.24	2.86
10	0.24	2.86
11	1.94	23.19
12	0.59	8.44
13	0.59	7.77
14	1.44	10.63
15	0.27	3.05
16	0.75	14.43
17	1.35	12.8
18	1.56	14.9
19	0.85	8.88

Number of objects	20	18	19
Block size	337.847 sqr mm	338.745 sqr mm	338.745 sqr mm
Average Perimeter	9.422 mm	6.063 mm	9.983 mm
Average size	1.227 sqr mm	0.650 sqr mm	0.914 sqr mm
Average Feret	1.846 mm	1.324 mm	1.81 mm
Density of objects	0.05920 sqr mm	0.05314 / sqr mm	0.0738 / sqr mm
Total area size	24.544 sqr mm	11.698 sqr mm	17.999 sqr mm
Percentage cover	7.27%	3.45%	5.30%

Non-responder R5 CA Collagen type VI			Responder R1 EMEM Collagen type VI			Responder R1 CYC Collagen type VI		
Number	**Area**	**Perimeter**	**Number**	**Area**	**Perimeter**	**Number**	**Area**	**Perimeter**
1	0.69	7.41	1	0.83	7.52	1	0.6	6.57
2	0.57	6.7	2	3.99	38.18	2	0.26	4.48
3	2.1	20.47	3	0.61	4.58	3	0.2	1.85
4	0.2	2.93	4	0.12	2.79	4	1.07	9.2
5	0.3	4.83	5	0.13	1.62	5	0.28	4.32
6	0.95	7.36	6	0.18	3.49	6	0.47	4.46
7	0.56	6.36	7	0.2	2.19	7	1.16	12.81
8	0.58	5.23	8	0.19	3.35	8	0.24	2.35
9	2.02	20.68	9	0.14	4.16	9	0.33	3.26
10	1.23	11.44	10	0.25	2.78	10	0.42	6.35
11	3.52	27.8	11	0.29	3.04	11	0.98	8.59
12	0.26	3.23	12	0.15	1.58	12	1.89	14.54
13	0.9	6.93				13	0.21	3.08
14	0.14	2.72				14	1.31	11.86
15	2.58	31.27				15	0.54	6.3
16	0.26	3.09				16	0.23	3.21
17	0.22	3.57				17	5.87	38.59
18	0.25	3.31				18	0.09	1.41
19	0.72	9.1				19	4.39	21.82
20	0.19	2.05				20	0.18	1.94
21	0.19	1.89				21	0.28	3.15
22	0.09	1.42				22	0.24	2.87
23	0.41	4.24				23	0.09	1.55

Number of objects	23	12	23
Block size	338.745 sqr mm	337.485 sqr mm	338.745 sqr mm
Average Perimeter	8.435 mm	6.274 mm	7.59 mm
Average size	0.822 sqr mm	0.589 sqr mm	0.928 sqr mm
Average Feret	1.81 mm	1.524 mm	1.508 mm
Density of objects	0.0679 / sqr mm	0.03556 / sqr mm	0.06790 / sqr mm
Total area size	18.912 sqr mm	7.068 sqr mm	21.346 sqr mm
Percentage cover	5.58%	2.09%	6.30%

Responder R1
CA
Collagen type IV

Number	Area	Perimeter
1	0.45	4.22
2	0.39	2.89
3	0.46	3.71
4	0.9	4.32

Responder R1
CA
Collagen type VI

Number	Area	Perimeter
1	1.99	15.99
2	0.35	5.98
3	0.82	5.07
4	0.33	3.16
5	1.66	10.86
6	0.37	2.35
7	0.29	2.37
8	0.4	5.98
9	0.95	7.17
10	0.96	7.66
11	5.94	41.14
12	1.12	10.68
13	1.74	13.04
14	2.38	17.98
15	0.18	1.54
16	0.65	6.65
17	1.13	7.97
18	1.48	14.72
19	1.16	7.21
20	1.33	4.88

Responder R2
EMEM
Collagen type IV

Number	Area	Perimeter
1	2.16	12.67
2	2.52	29.91
3	0.81	5.15

Number of objects	4	20	3
Block size	339.285	338.745 sqr mm	339.644 sqr mm
Average Perimeter	3.785	9.545 mm	15.908 mm
Average size	0.549 sqr mm	1.261 sqr mm	1.83 sqr mm
Average Feret	1.057 mm	1.928 mm	3.187 mm
Density of objects	0.01179 sqr mm	0.07675 / sqr mm	0.00883
Total area size	2.196 sqr mm	25.245 sqr mm	5.49 sqr mm
Percentage cover	0.65%	7.45%	1.62%

Responder R2
EMEM
Collagen type VI

Number	Area	Perimeter
1	2.389	13.05
2	8.5	25.07
3	4.13	24.19
4	1.18	11.8
5	0.23	2.96

Responder R2
CYC
Collagen type IV

Number	Area	Perimeter
1	1.51	10.93
2	0.38	6.95
3	0.63	12.05
4	2.59	17.48

Responder R2
CYC
Collagen type V

Number	Area	Perimeter
1	0.23	2.48
2	0.24	2.49
3	0.27	2.46
4	0.36	2.97

Number of objects	5	4	4
Block size	338.745 sqr mm	338.745 sqr mm	338.745 sqr mm
Average Perimeter	15.414 mm	11.854 mm	2.6 mm
Average size	3.288 sqr mm	1.279 sqr mm	0.275 sqr mm
Average Feret	3.013 mm	2.322 mm	0.724 mm
Density of objects	0.01475 / sqr mm	0.01181 / sqr mm	0.01176 /sqr mm
Total area size	16.44 sqr mm	5.118	1.099 sqr mm
Percentage cover	4.85%	1.51%	0.32%

Responder R2
CYC
Collagen type VI

Number	Area	Perimeter
1	1.05	8.23
2	0.53	9.75
3	3.87	21.46
4	6.26	51.85
5	0.28	3.14
6	0.24	2.61
7	2.83	32.39
8	3.13	25.72
9	2.53	14.39
10	1.07	11.82
11	2.22	12.68
12	0.58	7.46
13	0.25	2.64
14	0.77	8.49
15	0.34	4
16	0.4	5.03
17	0.45	4.89
18	0.96	9.01
19	1.42	14.59

Responder R2
CA
Collagen type V

Number	Area	Perimeter
1	0.42	5.03
2	0.37	3.08
3	0.23	2.21
4	0.39	3.41
5	0.23	2.37
6	0.3	2.56
7	1.32	10.42

Responder R2
CA
Collagen type VI

Number	Area	Perimeter
1	0.32	3.56
2	11.81	78.38
3	1	8.76
4	0.5	5.47
5	0.79	9.11
6	0.44	3.71
7	1.57	12.68
8	0.29	3.29
9	0.63	9
10	0.18	2.36
11	0.2	3.2
12	1.54	15.42
13	0.45	4.03
14	1.21	12.79
15	1	12.75
16	0.77	12.25
17	0.38	4.07
18	2.31	16.42
19	1.07	8.81

Number of objects	19	7	19
Block size	338.745 sqr mm	338.745 sqr mm	337.847 sqr mm
Average Perimeter	13.165	4.155 mm	11.899 mm
Average size	1.512 sqr mm	0.465 sqr mm	1.393
Average Feret	2.033 mm	1.128 mm	2.102
Density of objects	0.06495 / sqr mm	0.02066 / sqr mm	0.05624 / sqr mm
Total area size	29.185 sqr mm	3.256 sqr mm	26.475 sqr mm
Percentage cover	8.62%	0.96%	7.84%

Responder R3
CA
Collagen type V

Number	Area	Perimeter
1	0.18	4.12
2	0.02	1.07
3	0.64	9.9
4	0.12	2.92
5	1.28	9.14
6	0.34	3.09
7	0.56	6.11
8	0.2	5.44
9	1.34	10.64
10	0.34	2.82
11	3.45	11.49
12	0.8	7.64
13	0.5	3.89
14	1.01	6.52
15	0.66	5.58
16	0.65	5.79
17	0.39	4.13

Responder R3
CA
Collagen type VI

Number	Area	Perimeter
1	0.32	3.53
2	0.97	13.94
3	4.5	30.64
4	0.24	3.07
5	3.08	24.91
6	0.52	5.13
7	1.44	12.84
8	6.25	46.45
9	0.19	1.98
10	0.72	6.02
11	0.32	2.76
12	0.38	3.11
13	0.41	5.75
14	1.41	13.53
15	1.12	8.75
16	2.95	14.7
17	0.35	4.85
18	2.02	20.17

Number of objects	17	18
Block size	338.745 sqr mm	339.644 sqr mm
Average Perimeter	5.899 mm	12.335 mm
Average size	0.734 sqr mm	1.51 sqr mm
Average Feret	1.555 mm	2.383 mm
Density of objects	0.05019 / sqr mm	0.053 / sqr mm
Total area size	12.471 sqr mm	27.186 sqr mm
Percentage cover	3.68%	8.00%

Responder R3
EMEM
Collagen type VI

Number	Area	Perimeter
1	0.71	8.56
2	0.31	4.01
3	1.97	11.53
4	0.18	2.26
5	0.47	5.71
6	1.05	7.45
7	0.32	4.55
8	3.4	12.37
9	0.39	6.73
10	2.83	17.08
11	0.71	8.4
12	1.32	11.15
13	0.23	2.2
14	2.67	19.65
15	0.43	5.48

Responder R3
CYC
Collagen type V

Number	Area	Perimeter
1	0.23	2.52
2	0.49	3.88
3	0.44	5.15
4	0.15	1.99
5	0.12	1.3
6	0.34	2.91
7	0.14	1.53

Responder R3
CYC
Collagen type VI

Number	Area	Perimeter
1	0.37	5.43
2	0.27	2.53
3	1.99	24.03
4	2.07	15.32
5	0.24	5.09
6	4.27	18.09
7	0.15	2.04
8	1.83	15.73
9	2.73	21.1
10	0.12	1.53
11	0.83	6.59
12	0.69	6.68
13	1	8.04
14	0.19	2.29
15	1.69	11.71
16	0.74	6.84
17	1.88	17.32
18	2.82	17.02

Number of objects	15	7	18
Block size	338.745 sqr mm	338.745 sqr mm	337.485 sqr mm
Average Perimeter	9.075 mm	2.755 mm	10.410 mm
Average size	1.133 sqr mm	.273 sqr mm	1.327 sqr mm
Average Feret	1.869 mm	.787 mm	1.627 mm
Density of objects	0.04428 / sqr mm	0.02072 / sqr mm	0.0526 / sqr mm
Total area size	17.002 sqr mm	1.914 sqr mm	23.880 sqr mm
Percentage cover	5.02%	0.57%	7.08%

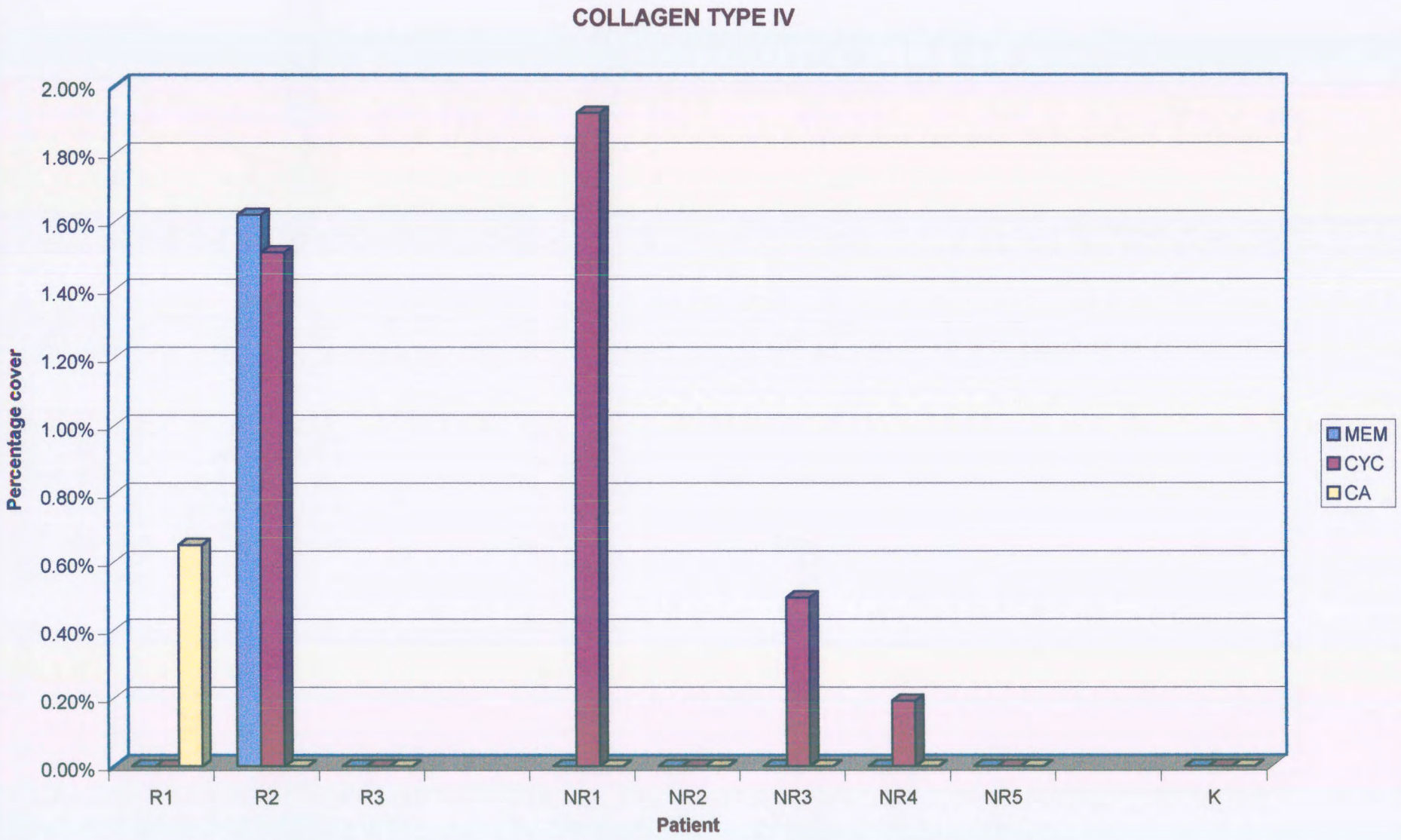

Figure E.1: Percentage Collagen Type IV observed with all 3 groups after treatment with both drugs.

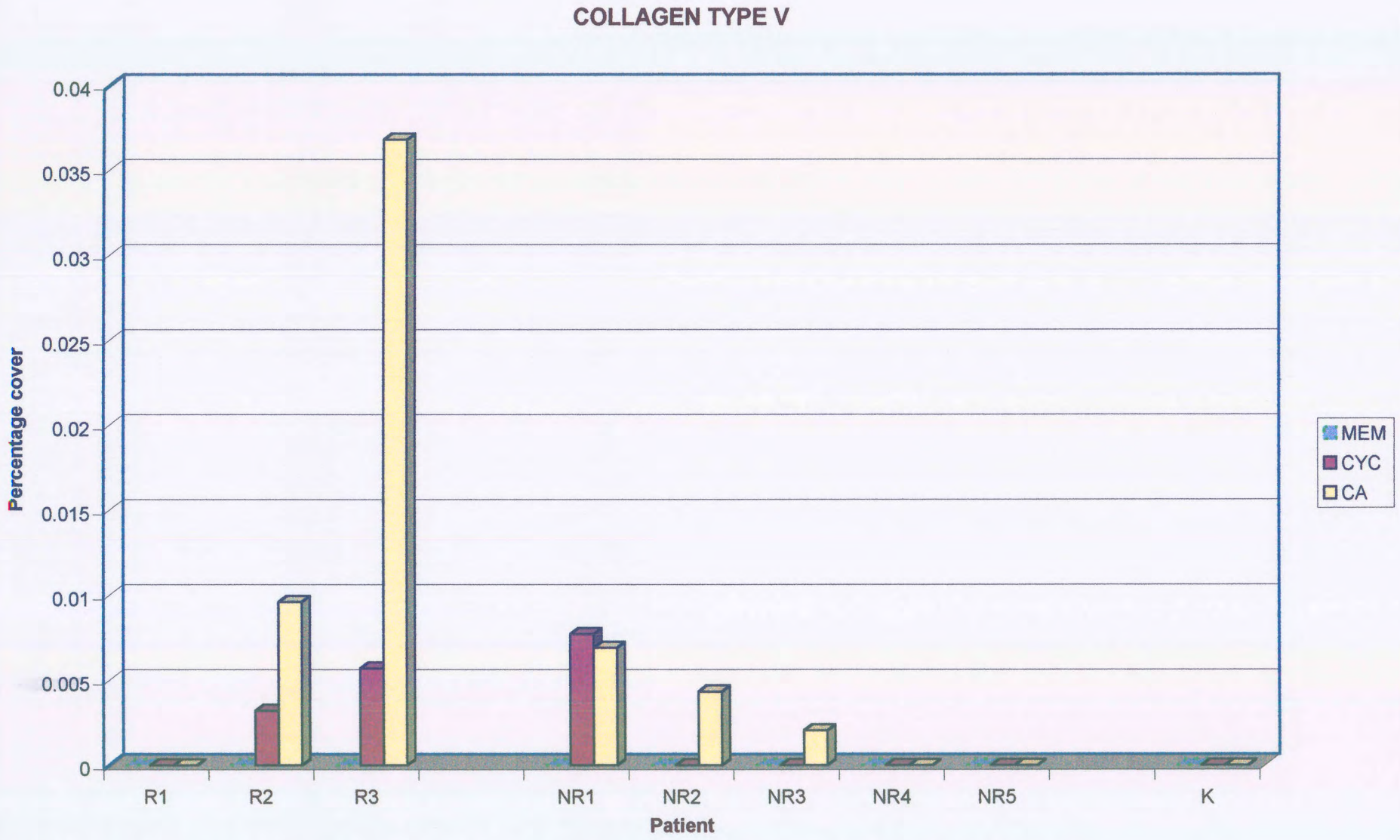

Figure E.2: Percentage Collagen Type V observed with all 3 groups after treatment with both drugs.

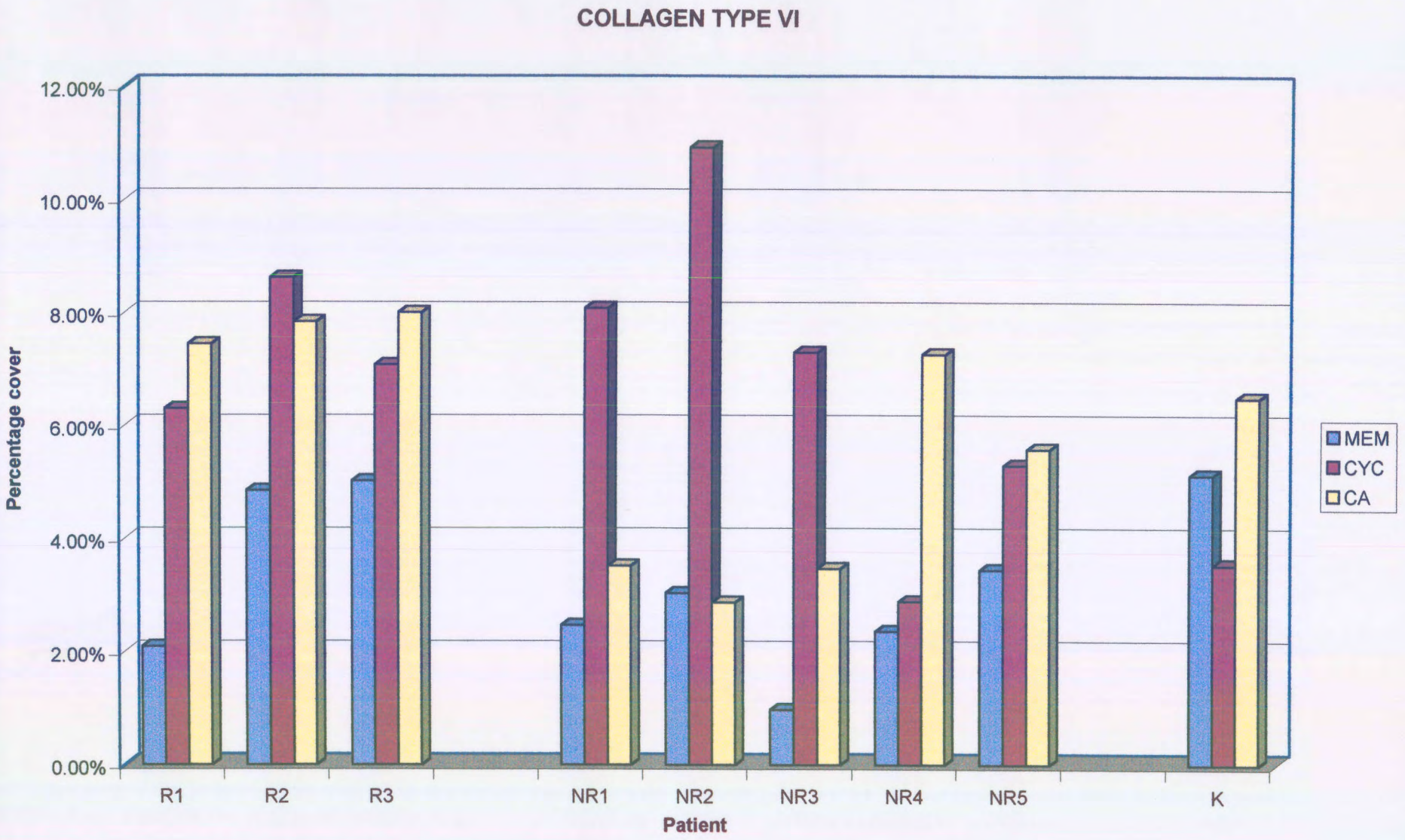

Figure E.3: Percentage Collagen Type VI observed with all 3 groups after treatment with both drugs.