The role of HLA-B27 in ankylosing spondylitis.

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(1) HLA-B27 in ankylosing spondylitis and uveitis.

In 1973, two seminal observations were published, one from the Westminster Hospital in London and the other one from Terasaki's group in Los Angeles, that over 95% of patients suffering from ankylosing spondylitis carried the major histocompatibility marker HLA-B27 whilst it was present in only 8% of the general populationon (Schlosstein, et al., 1973). Clearly here was a medical puzzle that required some form of explanation.

The first study from the Westminster Hospital, showed that out of a series of 75 controls, 4% had HLA-B27 but 72 patients with ankylosing spondylitis out of 75, that is 96%, had the HLA-B27 antigen. This was published in "Nature" (Caffrey and James, 1973) and the second report appeared in the "Lancet", 3 months later (Brewerton et al., 1973a).

Some 30% of ankylosing spondylitis develop an episode of uveitis during their lifetime. The Westminster group investigated a group of ophthalmological patients with acute anterior uveitis.

HLA-B27 was identified in 26 out of 50 patients suffering from acute anterior uveitis but only 2 out of the control subjects possessed the HLA-B27 antigen (Brewerton et al., 1973b). Here was definite evidence that not only ankylosing spondylitis but also uveitis travelled together with this genetic marker HLA-B27.

Dr. D.C.O. James, the haematologist involved in the discovery of HLA-B27 at the Westminster Hospital, came to the immunology lectures, at Queen Elizabeth College, now part of King's College, to discuss this problem. We had previously shown that "high" and "low" immune responses in inbred animals could be explained by "molecular mimicry" between H-2, which is the mouse equivalent of human HLA and an external, environmental antigen (Ebringer and Davies, 1973).

The hypothesis was proposed that there was probably some form of "molecular mimicry" between an unknown microbe and HLA-B27. This was based on the precedent of rheumatic fever and Sydenham's chorea being caused by anti-*streptococcal* antibodies following an upper respiratory infection such as tonsillitis.

Dr. Bill Boyle, the chief rheumatologist at the Middlesex Hospital was approached with the suggestion that this could be investigated in an "Ankylosing Spondylitis Research Clinic". He gave his approval and supported the clinic with financial resources. The immunological studies were carried out at Queen Elizabeth College and the clinical studies at the Middlesex Hospital. Some 900 ankylosing spondylitis patients were seen in the clinic between 1975 and 2002, when the clinic was closed.

(2) Reiter's disease and HLA-B27.

Reiter's disease or "la maladie de Fiessinger-Leroy" as it is known in France, is a condition associated with a triad of symptoms; urethritis, conjunctivitis and arthritis. It was described following the unhygienic conditions under which soldiers were living in the trenches during the First World War and succumbed from this disease. The implication was that it was a condition associated with some bacterial infection.

In a further study from the Westminster Hospital, HLA-B27 was found in 25 out of 33 patients with Reiter's disease (76%), in 9 out of 33 patients with non-specific urethritis but in only 2 out of 33 controls (Brewerton et al., 1973c).

In a Finnish study, HLA-B27 was identified in 43 out of 49 patients with *Yersinia* arthritis (88%) and in 36 out of 40 Reiter's disease patients (90%) but HLA-B27 is found in only 14% of the general Finnish population (Aho et al., 1974).

(3) The concept of "Gram negative reactive arthritis and HLA-B27.

The three main Gram-negative microorganisms associated with "reactive arthritis" are *Salmonella, Shigella* and *Yersinia* and the majority of affected patients are carriers of HLA-B27 (Aho et al., 1973).

Several epidemiological studies have been carried out that clearly establish a temporal relationship between a colonic infection and the subsequent "reactive arthritis" in a genetically susceptible host.

(A) Paronen's study:

In 1944, during the Russo-Finnish war, a *Shigella* dysentery epidemic occurred on the Karelian front, affecting 150,000 soldiers and Paronen reported 344 cases of Reiter's disease which developed after the gastroenteritis (Paronen, 1948). Over 20 years later, 100 of the original 344 were traced and 32 were found to have to have ankylosing spondylitis or sacro-iliitis (Sairanen et al., 1969).

Six years later, after Paronen had died and when the relevance of tissue typing had become recognized, 16 out of the 32 patients with ankylosing spondylitis or sacroiliitis were further traced and 15 (or 94%) were found to possess HLA-B27 (Sairanen and Tiilikainen, 1975).

(B) The "USS Little Rock" epidemic:

In the notorious post-dysenteric cases of Reiter's disease which occurred among the members of the crew of the "USS Little Rock", the "reactive arthritis" developed predominantly in individuals carrying the appropriate HLA antigens. An epidemic of *Shigella* gastroenteritis which affected about half the sailors was followed by 9 cases of Reiter's disease and all of these had suffered from gastroenteritis (Noer, 1966).

Subsequently 8 out of the original 9 individuals with Reiter's disease were traced; 7 were HLA-B27 positive and one carried HLA-B7, an allele which belongs to the B7-CREG group and crossreacts with HLA-B27 (Calin and Fries, 1976).

(C) *Salmonella* reactive arthritis in Finland:

In a study of 63 Finnish patients, diagnosed with "*Salmonella* reactive arthritis", between 1970-1986, 95% had abdominal pains and stool cultures were positive for *Salmonella* strains in 93% of patients.

Subsequently low back pain occurred in 44% of patients but HLA-B27 was present in 88% of the patients. In a follow-up of 50 of those patients, 8 had developed chronic spondyloarthropathy and 6 out of 44 patients had developed radiological evidence of

sacro-iliitis (Leirisalo-Repo et al., 1997).

(3) Geography of HLA-B27.

HLA-B27 is present throughout the world with some notable exceptions.
HLA-B27 is virtually absent among the genetically, unmixed, native populations of South America, Australia, African Bantus and Sans Bushmen.
In striking contrast, there is a very large prevalence of HLA-B27 among the native peoples of circumpolar arctic regions of Eurasia and North America (Khan M, 1988).
The frequency of HLA-B27 in Canadian Inuit population is 25%, among the Inuit-Eskimos of Greenland it is 30% and among the Siberian Chukchi Inuits it is 40% (Khan, 1998).
However the highest frequency of HLA-B27 is among the Haida Indians living on Queen Charlotte Island of British Columbia where it approaches 50% and definite ankylosing spondylitis has been reported to occur in 4% of the adult male Haida population (Gofton, 1980).

A striking South-to-North gradation in the frequency of HLA-B27 occurs in European populations: Southern Europeans 2 – 6%, Western Europeans 6 – 9%, Slavic populations 7 – 14% and Ugro-Altaic (Finland, Estonia, Hungary) populations 12 – 16%.
The frequency of HLA-B27 in northern Norway and northern Sweden is 10 – 16% and that of ankylosing spondylitis 1.4% but whether this reflects an admixture of Lapland populations is unclear.
The risk for ankylosing spondylitis in HLA-B27 positive first degree relatives of ankylosing spondylitis is much greater than among the HLA-B27 population at large.

(4) HLA-B27 as the predisposing gene.

The following features appear to favour a direct role for HLA-B27 in the pathogenesis of ankylosing spondylitis:

(a) The prevalence of ankylosing spondylitis depends on the racial background of the population and follows to a large extent the distribution of HLA-B27 in the general population.

(b) The association between HLA-B27 and ankylosing spondylitis

transcends racial and geographic barriers.

(c) In families with multiple cases of ankylosing spondylitis, one observes that the disease almost invariably segregates with HLA-B27.

(d) No stronger association has been observed with any other HLA group belonging to A, B, C or DR loci.

(e) HLA-B27 negative ankylosing spondylitis patients have a somewhat later age of onset and have a lower incidence of uveitis compared to HLA-B27 positive patients (Linssen, 1990).

(5) Molecular mimicry and rabbit immunisations.

The hypothesis was proposed that an external, infectious agent possessed molecular structures which stereochemically resembled HLA-B27.

Rabbit A was immunised with lymphocytes from 83 HLA-B27 positive healthy bone-marrow donors attending the "Westminster Hospital Tissue typing Unit". The cells were separated and injected in complete Freund's adjuvant on 12 occasions over a period of 3 months.
Rabbit B was immunised with lymphocytes from 3 patients with ankylosing spondylitis all having the tissue type HLA-B27.

Serum from rabbit A produced precipin lines against 3 microorganisms out of 31 tested: *Enterobacter, Klebsiella* and *Yersinia.*
Serum from rabbit B produced precipin lines against *Shigella* and *Klebsiella*.

Rabbits A and B immunised with HLA-B27 lymphocytes showed haemagglutinating activity against sheep red cells coated with *Klebsiella* lipolysaccharide and these elevations were statistically significant when compared to serum obtained from the same rabbit before immunisation (Welsh et al., 1980).

Ankylosing spondylitis patients were examined for the presence of the 4 microbes detected in the immunological studies: *Enterobacter, Klebsiella, Yersinia* and *Shigella.* Only *Klebsiella* was detected in fecal samples from active ankylosing spondylitis patients and further studies were confined to this microorganism (Ebringer et al., 1976).

(6) Antibodies to *Klebsiella* in ankylosing spondylitis.

Antibody levels against *Klebsiella* microbes have been measured in ankylosing spondylitis patients from 16 different countries by a variety of of techniques: Widal agglutination, ELISA, immunoblotting and immunofluorescence. In each case the results were similar in that active ankylosing spondylitis patients had elevated levels of antibodies to *Klebsiella* microbes (Table 1). Active patients were defined as those having biochemical evidence of inflammation, namely an elevated erythrocyte sedimentation rate (ESR) and a rise in C-reactive protein levels (Cowling et al., 1980a).

On 3 separate occasions, antibodies were measured simultaneously against *Klebsiella* and *Proteus* microbes in active ankylosing spondylitis and rheumatoid arthritis patients from England (Ebringer et al., 1985), the Netherlands (Blankenberg-Sprenkels et al., 1998) and Japan (Tani et al., 1997). In each case the results were the same in that ankylosing spondylitis patients had antibodies against *Klebsiella* but not against *Proteus,* while rheumatoid arthritis patients had antibodies against *Proteus* but not against *Klebsiella,* and controls had low titres against both microbes.

(7) Molecular mimicry and *Klebsiella* enzymes.

An amino acid homology QTDRED, found in residues 72-77 of HLA-B27 has been identified which has molecular identity with residues 188-193 of *Klebsiella pneumoniae* nitrogenase reductase enzyme (Schwimmbeck et al., 1987) but for the enzyme to be produced an extremely low level of fixed nitrogen is required and a temperature of 32ºC, conditions which are not achieved in the human gut.

Further computer analyses were carried out.

(A) *Klebsiella* pullulanase-D and HLA-B27:

A tetramer DRDE, present in the debranching enzyme *Klebsiella* pullulanase-D secretion protein (residues 596-599) was identified as having homology with HLA-B27 (Figure 1).

Antibodies to 15-mer peptides of the pul-D molecule, containing in the middle the DRDE sequence were found to be elevated in 97 ankylosing spondylitis patients (Fielder et al., 1995).

(B) *Klebsiella* pullulanase-A and collagen sequences:

Another component of the debranching enzyme complex, namely pullulanase-A was found to have collagen like sequences which were crossreacting with collagen I, collagen III and collagen IV (Table 2).

The levels of cross-reactive antibodies to collagens type I and type IV in the sera of ankylosing spondylitis patients have been found to be elevated and this suggests they have a role to play in the pathogenesis of enthesitis, spondylitis and uveitis (Fielder et al., 1995).

Type I collagen is found predominantly in tendons and bone whilst type IV collagen is present in basement membranes, basal lamina, retina, cornea and uvea.

Muscle stiffness and often accompanied by tenderness is a well recognised feature of ankylosing spondylitis. The source of these characteristic symptoms has long been attributed to joint inflammation and to inflammation at the site of attachment of ligaments or tendons to bone, to the enthesis (Ball 1971).

The dramatic relief obtained by exercise, as compared to the slower less pronounced relief, produced in other inflammatory arthropathies, could indicate the involvement of the muscle itself.

A higher serum creatine phosphokinase level was found in a group of ankylosing spondylitis patients when compared to healthy subjects and iso-enzyme studies confirmed that muscle was the source of the enzyme (Calin, 1975).

(8) The proposed model for the pathogenesis of ankylosing spondylitis.

The pathogenesis of ankylosing spondylitis, according to the molecular mimicry hypothesis can be divided into five stages. It is relevant to note that in this pathogenetic model there are no bacterial fragments or antigens at sites of inflammation, around the entheses, in the spine or the uvea.

The 5 stages are as follows:

Stage 1: Infection by *Klebsiella* microbes:

Antibodies to *Klebsiella* microorganisms in patients with ankylosing spondylitis have been reported from 16 different countries (Table 1). The world wide distribution of increased *Klebsiella* antibodies in ankylosing spondylitis patients would strongly support the hypothesis that exposure to this microbe occurs in this disease. In this primary stage antibodies are produced against *Klebsiella* antigens.

Stage 2: Production of anti-DRDE antibodies and anti-collagen antibodies:

The various antigenic molecules present on the surface of *Klebsiella* microorganisms including capsular polysaccharides or enzymes produced by these microbes such as pullulanase, may all be triggering the disease development in ankylosing spondylitis.

The anti-DRDE antibodies evoked by pulD and the anti-collagen I, III and IV antibodies evoked by pulA are the main pathogenic agents which are produced in the local lymph nodes draining the colon.

When these autoantibodies leave the lymph nodes and localise in the near-by related tissues through Batson's plexus, an anatomical connection has been made to the lumbar spine and associated joints.

Stage 3: Binding of anti-DRDE to HLA-B27 and anti-pulA antibodies binding to collagens I, III and IV:

If the anti-pulD and anti-pulA antibodies are present in sufficiently high concentrations, then two adjacent IgG molecules will activate the complement cascade, which will lead to a cytopathic event and the triggering of inflammation, with interleukin and cytokine production.

Thus the anti-bacterial antibodies are acting as autoantibodies and this makes ankylosing spondylitis an autoimmune disease (Wilson et al., 2003).

The use of biologicals at this stage, that is "stage 3", will limit the damage caused by the bacterially evoked autoantibodies.

However it is relevant to note that the disease can also be interfered with, by blocking or targeting the "stage 1" of the pathogenic pathway.

This could be done by reducing the quantities of *Klebsiella* bacteria in the colon by using antibiotics, such as sulphasalazine, moxifloxacin

(Ogrendik, 2007) or reducing the quantity of substrates on which these bacteria grow and proliferate.

Stage 4: Inflammation by autoantibodies produces enthesitis and uveitis:

The presence of anti-pulD antibodies will bind to the HLA-B27 chondrocytes and in presence of high concentration of antibodies and complement will cause tissue damage which in turn will set off localised inflammation.

The presence of anti-pulA antibodies will bind to collagens I and III found in the lumbar spine and also to collagen IV found in the basal membranes and the uvea and give rise to enthesitis, Crohn's like lesions in the small bowel and uveitis.

Stage 5: Repeated episodes of colonic *Klebsiella* infection will lead recurrent inflammation, fibrosis and eventually classical ankylosing spondylitis:

Repeated episodes of bacterial infection by *Klebsiella* bacteria in the colon will lead to waves of high titres of anti-*Klebsiella* antibodies which will produce intermittent but recurrent inflammation characterised by backache, morning muscle stiffness and occasionally uveitis with eventual development of classical ankylosing spondylitis.

(9) Importance for early diagnosis of ankylosing spondylitis.

Several years are required for the classical, clinical features of ankylosing spondylitis, particularly radiological evidence of sacro-iliitis to appear before the diagnosis can be established. Often, by the time the disease is diagnosed, the destructive pathological events have occurred and at this late stage it appears to be difficult to reverse the damaging effects of the disease.

It should be emphasized that association of inflammatory back pain together with certain other features such as enthesitis, arthritis and acute anterior uveitis, as well as the presence of HLA-B27 greatly increase the probability of "undifferentiated spondyloarthritis" to be considered as "ankylosing spondylitis" (Rudwaleit et al., 2004).

In a 10-year follow-up study of a group of probable ankylosing spondylitis patients, it was observed that the progression from "undifferentiated spondyloarthritis" to definite ankylosing spondylitis would occur after some 9 years (Mau et al., 1988).

The great majority of ankylosing spondylitis patients who carry HLA-B27 and who live in a relatively salubrious and urban environment have been infected by the commensal microbe *Klebsiella*, a microbe that does not cause overt gastro-enteritic symptoms as occurs with *Salmonella, Shigella* or *Yersinia* microorganisms, microbes known to be associated with "Gram-negative reactive arthritis".

(10) Growth of *Klebsiella* on dietary substrates.

It would appear that antibodies to the normal bowel commensal microbe *Klebsiella* are present in ankylosing spondylitis patients throughout the world.

Nearly all normal subjects fail to absorb appreciable amounts (5 – 20%) of starch in wheat flour, commonly present in bread and pasta, as assessed by oral hydrogen excretion studies, following a test meal.
Breath hydrogen concentration were measured for 5 hours after an overnight fast, followed by a test meal containing equal amounts of either sucrose, bread or pasta. Oral hydrogen is a measure of bacterial proliferation in the colon. The sucrose meal produced no increase in oral hydrogen since it would have been absorbed in the stomach and upper small bowel. However significant amounts of oral hydrogen were detected at 4 and 5 hours after a test meal with bread and pasta (Anderson et al., 1981).

It would appear that the quantities of carbohydrate substrates entering the large bowel across ileo-caecal junction are important in determining the number of *Klebsiella* microbes.
An important source of carbohydrate substrate comes from dietary starch. Dietary starch consists of approximately 20% amylose and 80% amylopectin. Amylose is a linear polymer consisting of α-(1-4) links between glucose residues and these can be readily hydrolysed by amylases present in digestive enzymes.
However, amylopectin is a branched polymer, consisting of linear sequences of amylose like chains linked by α-(1-4) bonds but for every 10 to 15 residues, there is a α-(1-6) side chain giving rise to a branched

structure (Figure 2). The problem is that digestive enzymes cannot break down the α-(1-6) links present in amylopectin.

The digestion of starch in the small bowel is limited by the inability of luminal digestive enzymes to break α-(1-6) bonds of amylopectin and thereby giving rise to "hard starch" which accumulates in the colon.

Klebsiella pullulanase is a powerful enzyme which degrades α-(1-6) amylopectin links, thereby providing more substrate for the growth of *Klebsiella* .

(11) Starch restriction in ankylosing spondylitis.

Since starch or amylopectin is a substrate for *Klebsiella* pullulanase a trial of starch restriction could be beneficial in patients with ankylosing spondylitis.

Double blind trials are clearly impossible to carry out since patients would be aware of what they are eating.

However objective criteria, such as erythrocyte sedimentation rates, C-reactive protein levels and total serum IgA are available to carry out such studies.

Serum IgA levels have been reported to be elevated in active ankylosing spondylitis patients (Cowling et al., 1980b)

(1) Open study with 21 healthy controls:

A group of 21 healthy subjects, namely University students and hospital staff attempted the "low starch diet" for a period of 8 weeks.

The "low starch diet" was defined as: "No bread, no potatoes, no cakes, no pasta and no rice".

To compensate for the calorie loss incurred in reducing starch intake, the healthy control subjects were advised to increase their intake of meat, fish, fruits and vegetables but excluding potatoes.

There were no restriction on their drinking habits, an important point with some of the University students.

Blood samples were taken at the beginning and end of the study. Total serum IgA and serum C-reactive protein was measured before and after the end of the trial.

Total serum IgA was considered to be an estimate of the quantity of bowel flora present in the colon, evoking an immune response.

Total serum IgA significantly dropped from 255 mg/dl at the start of the study to 212 mg/dl ($p<0.001$) by the end of 8 weeks (Figure 3).

The C-reactive protein levels were also measured before and after the end of the diet. All control subjects had zero C-reactive protein levels before and after the completion of the diet

(2) Ankylosing spondylitis patients partaking in the diet study:

A group of 74 randomly chosen ankylosing spondylitis patients in both active and inactive stages of the disease were asked to participate in the "low starch diet" for 9 months.

There were 51 male and 13 female patients and the mean (± standard error) age was 41.0 ± 1.4 years (Range 22- 67 years).

The ankylosing spondylitis patients were divided into 2 groups:
36 patients had an erythrocyte sedimentation rate above 15 mm/hour and were considered to be biochemically active and the remainder who had an erythrocyte at 15 mm/hour or below at the start of the trial.

In the active patients the mean erythrocyte sedimentation rate at the onset of the trial was 38.8 ± 3.1 mm/hour and at the end it had dropped to 24.3 ± 2.4 mm/hour ($t = 6.76$, $p < 0.001$) (Figure 4).

In the inactive group, there was no significant difference in the levels of erythrocyte sedimentation rates before and after the end of the trial (Ebringer et al., 1985).

These results involving readily available serological and biochemical criteria, indicate that at a "low starch diet" can be objectively studied in patients with ankylosing spondylitis.

The fact that many ankylosing spondylitis patients on the "low starch diet" reported clinical benefits does not detract from the objective results achieved in such studies.

Further investigations are required to combine the use of "biologicals" with "low starch diets" in the treatment of ankylosing spondylitis.

(12) Conclusions.

The link between HLA-B27 and "Gram negative arthritis" as well as urban ankylosing spondylitis has been documented in many studies.

Molecular mimicry between HLA-B27 and several Gram negative microbes, such as *Salmonella, Shigella, Yersinia* and *Klebsiella* provides an important clue to the pathogenesis of these diseases.

The fact that *Klebsiella* microbes possess a powerful debranching enzyme for starch substrates, should be utilised in implementing novel approaches in the treatment of ankylosing spondylitis.

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Table 1.
Countries showing antibodies to *Klebsiella* in ankylosing spondylitis patients.

Number	Country	Year of study	Reference
1.	England	1983	Trull et al.
2.	USA	1987	Schwimbeck et al.
3.	Scotland	1988	Cooper et al.
4.	Russia	1989	Nassonova et al.
5.	Slovakia	1989	Mateicka et al.
6.	China	1993	Yuan et al.
7.	Germany	1994	Sahly et al.
8.	Finland	1994	Maki-Ikola et al.
9.	Spain	1994	Collado et al.
10.	Turkey	1996	Ardicoglu et al.
11.	Japan	1997	Tani et al.
12.	Sweden	1997	Maki-Ikola et al.
13.	Taiwan	1998	Chou et al.
14.	Mexico	1998	Cancino-Diaz et al.
15	Netherlands	1998	Blankenberg et al.
16.	India	2002	Madhavan et al.

Table 2.

Comparison of amino acid sequence homologies between *Klebsiella* pullulanase (pulA) and collagens I, III and IV (G= glycine, X = any other amino acid, P = proline, A = alanine, D = aspartic acid, E = glutamic acid).

Protein	Residues	Amino acid sequence
pulA	**11- 29**	**GXP-GXP-GXP-GXP-GXP-GXP**
collagen I		**GXP-GXP-GAD-GPA-GXP-GXP**
collagen III		**GXP-GXP-GXP-GXP-GXP**
collagen IV		**GAE-GXP-GXP-GXP**

Legends to Figures.

Figure 1.

Molecular similarities between peptides from nitrogenase reductase and pullulanase enzymes compared to HLA-B27 molecule (With permission).

Figure 2.

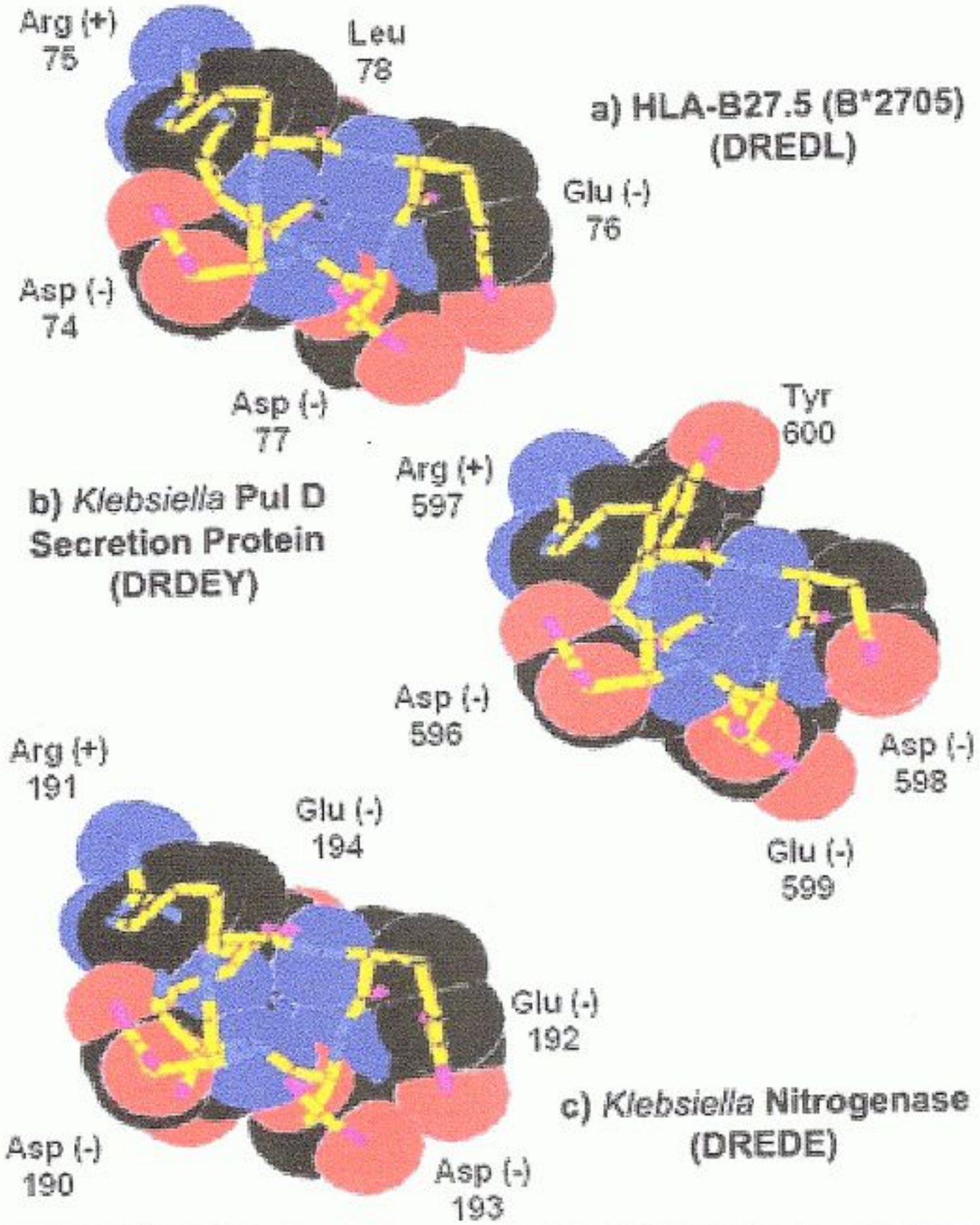
Klebsiella* pullulanase is a powerful debranching enzyme that breaks down the α (1-6) links in amylopectin and thereby provides carbohydrate substrates for the growth of colonic *Klebsiella.

Figure 3.

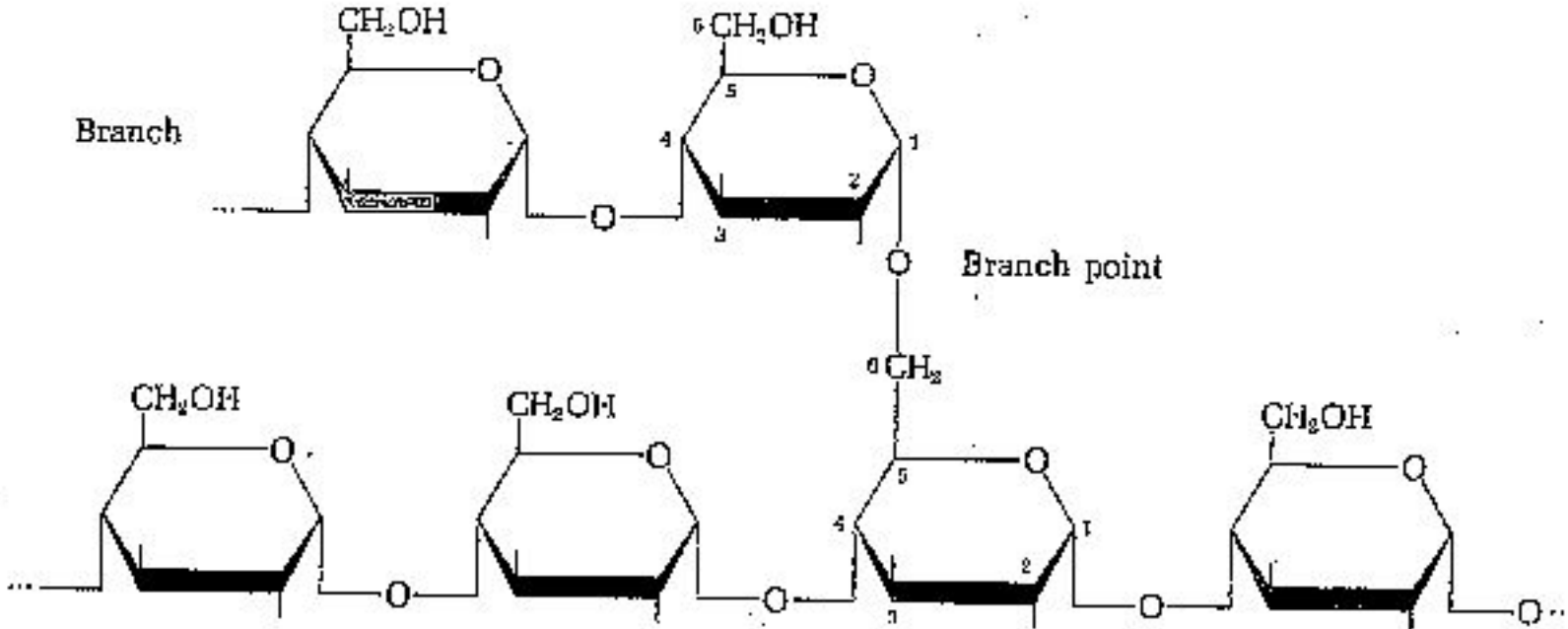
Total serum IgA concentrations measured in 21 healthy control subjects, before and after an 8-week "low starch diet" (With permission).

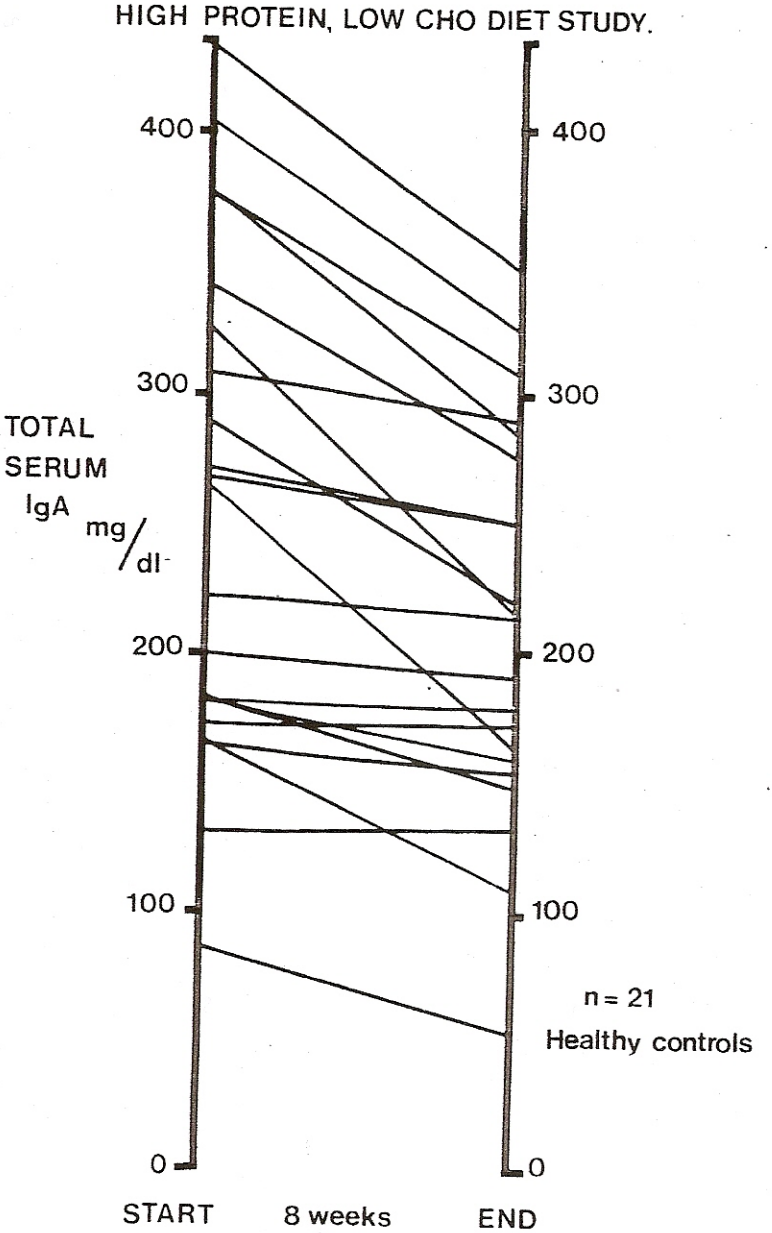
Figure 4.

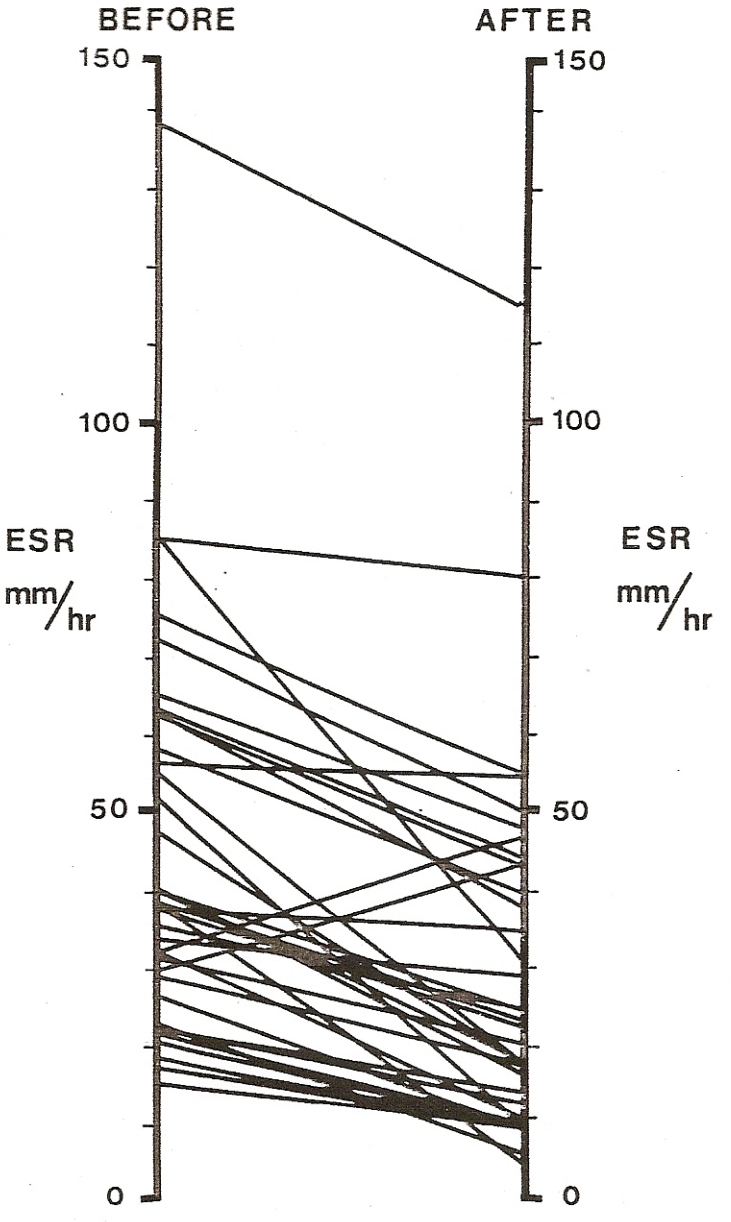
Erythrocyte sedimentation rate before and after 9 months on a "low starch diet" in 36 active ankylosing spondylitis patients (With permission).


Arg (+)
75
Leu
78
a) HLA-B27.5 (B*2705)
(DREDL)
Glu (-)
76
Asp (-)
74
Asp (-)
77
b) Klebsiella Pul D
Secretion Protein
(DRDEY)
Tyr
600
Arg (+)
597
Asp (-)
596
Asp (-)
598
Glu (-)
599
Arg (+)
191
Glu (-)
194
Glu (-)
192
Asp (-)
190
Asp (-)
193
c) Klebsiella Nitrogenase
(DREDE)

An $\alpha(1 \longrightarrow 6)$ branch point in amylopectin




HIGH PROTEIN, LOW CHO DIET STUDY.
400
300
200
100
0
400
300
200
100
0
TOTAL
SERUM
IgA
mg/dl
n = 21
Healthy controls
START
8 weeks
END


BEFORE
AFTER
150
150
100
100
ESR
mm/hr
ESR
mm/hr
50
50
0
0