CHAPTER 2
Insulin, Proinsulin and CAD

2.1. Introduction

Glucose intolerance and hyperinsulinemia are common metabolic aberrations occurring in diabetic and non-diabetic subjects with premature CAD (Bavenholm, et al., 1995a); but the pathogenic mechanisms underlying this relationship is still ambiguous.

![Subunit A and Subunit B](image1)

![Secondary and Primary Structure](image2)

**Figure 7**: (a) Secondary structure of Insulin, (b) Primary structure of Proinsulin

Insulin resistance or hyperinsulinemia is the only physiologic abnormality that can lead to the clustering of abnormalities comprising of hyperglycemia, atherogenic lipoprotein profile (high triacylglycerol and low HDL concentrations), essential hypertension, pro-coagulant and proinflammatory states, all of which increase the risk of cardiovascular disease (CVD). Values of insulin-mediated glucose disposal vary continuously throughout a population of apparently healthy persons, and a difference of $\geq 600\%$ exists between the most insulin-sensitive and the most insulin-resistant persons. About 50% of this inconsistency can be ascribed...
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to disparity in adiposity (25%) and fitness (25%), with the remaining 50% probably inherited (Reaven, 2006). Hyperinsulinaemia could also be proatherogenic in its own right (Stout, 1990), and studies have shown that increased concentration of insulin and proinsulin, either fasting or after a glucose load predicts incidence of CAD (Welborn and Wearne, 1979; Pyorala, 1979; Ducimetiere et al., 1980; Despres et al., 1996; Ruige, et al., 1998). Although hyperinsulinemia and/or IR have drawn considerable interest as possible risk factor for CAD, prior studies lack angiographic confirmation of CAD, have exhibited inconsistent results and moreover not demonstrated in South Indian population. Hence a current study was carried out to investigate the relation between insulin (Figure 7a), proinsulin (Figure 7b) in angiographically documented CAD and non-CAD subjects.

2.2. Background and Objectives of the Study

South Asians have a higher risk of CAD compared with the white population in Europe and the USA (Balarajan, 1995; Bhopal, et al., 1999). The increased risk of CAD has been attributed to a high insulin resistance (IR) in South Asians (Haffner, 1993b; Snehalatha, Ramachandran, Vijay and Viswanathan, 1994; Balarajan, 1995; Ramachandran, et al., 1997). Epidemiological data have shown a clustering of CAD risk factors in non-diabetic South Indians (Ramachandran, et al., 1998a), but IR alone fails to explain the clustering (Snehalatha, et al., 2000). There have been few studies from India investigating the role of hyperinsulinaemia or IR in CAD. There is also some disagreement on the association of proinsulin (PI) with cardiovascular risk factors in different ethnic groups (Haffner, 1993b; Yudkin, et al., 1997). In this study, we have evaluated the
association of IR, serum levels of insulin and proinsulin with CAD in diabetic and non-diabetic subjects in South Indian population.

Although western clinical (Stout, 1990) and population studies (Zavaroni, et al., 1989; Fontbonne, et al., 1991; Despres, et al., 1996) have shown association between hyperinsulinemia and CAD, others report conflicting results (Laakso, et al., 1991; Welin, et al., 1992; Raal, Panz, Pilcher and Joffe, 1999). However, much of the earlier studies probing on association between hyperinsulinemia and CAD had no angiographic confirmation of CAD except for a few (Mookherjee, Potts and Hill, 1984; Shinozaki, et al., 1996; Bressler, et al., 1996; Seibaek, et al., 1997; Takezako, et al., 1999). Hyperglycemia itself could be an alternative reason for the higher cardiovascular risk in patients with type-2 diabetes (Laakso, 1999). Post challenge hyperglycemia emerge to be strongly related with cardiovascular mortality (Barrett-Connor, et al., 1998; Shaw, et al., 1999; Tominaga, et al., 1999; Saydah, et al., 2001) or atherosclerosis (Temelkova-Kurkschiev, et al., 2000). However, these studies demonstrating an association between hyperglycemia and coronary atherosclerosis lacked angiographic verification of CAD.

Moreover, large-scale prospective epidemiological studies have indicated that hyperinsulinemia (Welborn and Wearne, 1979; Ducimetière, et al., 1980) and glucose intolerance are associated with the development of CAD. In addition, associations have been shown consistently between hyperinsulinemia, either fasting or after an oral glucose load, and established risk factors for cardiovascular disease, including abdominal obesity, hypertriglyceridemia, reduced HDL-C.
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concentration, and hypertension (Reaven, 2006). However, the mechanisms underlying the relations of serum insulin and proinsulin to CAD remain unclear.

It has been argued recently that a standard radioimmunoassay for insulin may overvalue the true insulin levels in type 2 diabetics and subjects with impaired glucose tolerance, due to cross-reaction with proinsulin (Temple, et al., 1990; Williams, et al., 1991). The question now arises whether the hyperinsulinemia previously demonstrated in diabetic and non-diabetic subjects who manifest CAD is to some extent interfered by proinsulin -like molecules and whether proinsulin and insulin are independent players in the development of coronary atherosclerosis and thrombosis. Therefore, we performed this study to investigate whether the presence or severity of angiographically documented coronary atherosclerosis is associated with the degree of hyperinsulinemia and/or insulin resistance and proinsulin, or alternatively, with hyperglycemia itself.

2.3. Literature Review

2.3.1. Role of Insulin in CAD

Diabetes mellitus and hypercholesterolemia are well established risk factors for CAD (Stern, 1994). Patients with CAD also have a high rate of glucose intolerance, hypertriglyceridemia (Hughes, Raval and Raftery, 1989; Wingard, Barret and Ferrara, 1995), low high density lipoprotein-cholesterol (HDL-C) levels, insulin resistance, and hyperinsulinemia, in addition to abdominal obesity and hypertension (Reaven, 2006). The observation that different ethnic groups have different rates of CAD suggests that these groups may differ with respect to
the frequency or nature of predisposing metabolic risk factors (Balarajan, 1995), and/or with respect to their susceptibility to these risk factors.

However, in these studies (Pyorala, 1979; Welborn, and Wearne, 1979; Ducimetiere et al. 1980; Cullen et al. 1983; Fontbonne et al. 1991; Pyorala, Miettinen, Laakso and Pyorala, 1998a), the predictive value of hyperinsulinemia diminished with extended follow-up; whereas two prospective population-based studies have also suggested that the association between hyperinsulinemia and increased risk of CAD may be independent (Despre’s, et al., 1996; Perry, et al., 1996). However, most studies in the 1990s have not reconfirmed that hyperinsulinemia is an independent risk factor for CAD (Liu, et al., 1992; Rewers, et al., 1992; Welin, et al., 1992; Ferrara, Barrett-connor and Edelstein, 1994; Orchard, et al., 1994; Yarnell et al. 1994; Kuusisto, Mykkanen, Pyorala and Laakso, 1995; Tuomilehto, et al., 1996; Lakka T.A, Lakka H.M and Salonen, 1996; Lindberg, et al., 1997; Folsom, et al., 1997; Ruige, et al., 1998). Few studies are available concerning the association between hyperinsulinemia and the risk of stroke. Hyperinsulinemia was not independently associated with the risk of stroke in the extended follow-up of the Helsinki Policemen Study (Pyorala, Miettinen, Laakso and Pyorala, 1998b). Neither did hyperinsulinemia predict stroke incidence in diabetic subjects without a previous stroke in a study of elderly men and women in Kuopio, Finland (Kuusisto, Mykkanen, Pyorala and Laakso, 1994a). Furthermore, few study groups have reported the relationship of hyperinsulinemia with all major CAD events and stroke (Pyorala, 1979; Pyorala, Miettinen, Laakso and Pyorala, 1998a; Pyorala, Miettinen, Laakso and Pyorala, 1998b). The
association of Insulin, Proinsulin, Proinsulin/Insulin ratio, and levels of lipoproteins to CAD risk in global and Indian population is reviewed in Table 1.

There is also ample evidence that hyperinsulinemia increases the incidence of dyslipidemia (Reaven, 1988) and hypertension (Haffner, et al., 1992a; Mykkanen, et al., 1994; Salonen, et al., 1998), signifying that hyperinsulinemia pave the way for these disorders in the etiopathogenesis, while the time order of the relationship between hyperinsulinemia and obesity is lacking clarity (Odeleye, de Courten, Pettitt and Ravussin, 1997; Lazarus, Sparrow and Weiss, 1998). Consequently, hyperinsulinemia could enhance the CAD risk through dyslipidemia, hypertension, and obesity instead of being risk factor for CAD by itself. Support for this view comes from several previous prospective studies (Casassus, et al., 1992; Yarnell, et al., 1994; Lakka, Lakka H.M and Salonen, 1996) which have concluded that, these abnormalities are interlinked with hyperinsulinemia and the risk of CAD. In contrast, in some prospective studies, although the relationship has been attenuated, hyperinsulinaemia has remained statistically significant even after adjustment for the confounding metabolic disorders (Despres, et al., 1996; Perry, et al., 1996; Pyorala, Miettinen, Laakso and Pyorala, 1998a).

Furthermore, The first MI attack occurs in 4.4% of Asian women and 9.7% of men at age less than 40 years, which is 2- to 3.5-fold higher than in the West European population and is third highest of all the regions studied worldwide (Yusuf, et al., 2004). Leeder, Raymond and Greenberg, (2004) estimate total years of life lost due to total cardiovascular disease (CVD) among the Indian men and
women aged 35-64 to be higher than comparable countries such as Brazil and China. These estimates are predicted to increase from 2000 to 2030, when the differences may become more marked (Leader, Raymond and Greenberg, 2004).

Table 1: Association of Insulin, Proinsulin, Insulin/Proinsulin ratio, and levels of lipoproteins to CAD risk in Global and Indian Population

<table>
<thead>
<tr>
<th>Author</th>
<th>Sample size</th>
<th>Insulin</th>
<th>Proinsulin</th>
<th>Insulin/Proinsulin Ratio</th>
<th>Lipoprotein Levels</th>
<th>CAD Risk</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fantin Sde, et al., (2011)</td>
<td>70</td>
<td>↑</td>
<td>-</td>
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<tr>
<td>Bhamidipati, et al., (2011)</td>
<td>4658</td>
<td>↑</td>
<td>-</td>
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<td>↑</td>
</tr>
<tr>
<td>Tanaka, et al., (2010)</td>
<td>582</td>
<td>↑</td>
<td>-</td>
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<tr>
<td>Acibucu, et al. (2010)</td>
<td>80</td>
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<td>-</td>
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<tr>
<td>Heck, et al., (2010)</td>
<td>24</td>
<td>↑</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>↑</td>
</tr>
<tr>
<td>Gui, et al., (2008)</td>
<td>546</td>
<td>↑</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>↑</td>
</tr>
<tr>
<td>Quadros, et al., (2006)</td>
<td>145</td>
<td>↑</td>
<td>-</td>
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<td>↑</td>
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<tr>
<td>Oh, Barrett-Connor and Wedick, (2002)</td>
<td>1456</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>-</td>
<td>↑</td>
</tr>
<tr>
<td>Matsumoto, et al., (2001)</td>
<td>33</td>
<td>↑</td>
<td>-</td>
<td>-</td>
<td>HDL-C↑, Lp(a)↑, TG↑</td>
<td>↑</td>
</tr>
<tr>
<td>Kuusisto, Lempiainen, Mykkanen and Laakso, (2001)</td>
<td>70</td>
<td>↑</td>
<td></td>
<td>-</td>
<td>-</td>
<td>↑</td>
</tr>
<tr>
<td>Ferrara and Goldberg, (2001)</td>
<td>45</td>
<td>↑</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>↑</td>
</tr>
<tr>
<td>Zhu, et al., (2000)</td>
<td>114</td>
<td>×</td>
<td>×</td>
<td>-</td>
<td>-</td>
<td>↑</td>
</tr>
<tr>
<td>Lakk, et al., (2000)</td>
<td>1521</td>
<td>↑</td>
<td>-</td>
<td>-</td>
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<td>↑</td>
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<tr>
<td>Matsumoto, et al., (1999)</td>
<td>98</td>
<td>↑</td>
<td>-</td>
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</tbody>
</table>
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An investigation into the relationship of insulin and other related biochemical parameters with coronary artery disease in a South Indian population

2.3.2. Role of Proinsulin in CAD

In the past decades, IR and CAD in type 2 DM patients have been associated with hyperinsulinemia as an independent risk factor (Welborn and Wearne, 1979; Arrants, 1994; Despres, et al., 1996). However, many studies failed to corroborate the negative impact of insulin on cardiovascular risk (Welin, et al., 1992; Ferrara, Barrett-Connor and Edelstein, 1994; Katz, et al., 1996). The reason

| Study                          | Sample Size | HDL-C | LDL-C | TG |?
|-------------------------------|-------------|-------|-------|----|-
| Gerstein, et al. (1999)       | 300         | ↑     | -     | ↑  |-
| Lempäinen, et al. (1999)      | 151         | ↑     | -     | ↑  |-
| Haffner, et al. (1998b)       | 985         | ↑     | ↑     | -  |-
| Katz, et al. (1996)           | 134         | ×     | ×     | -  |-
| Kahn, et al. (1995)           | 170         | ↑     | ↑     | ↑  |-
| Kuussisto, Mykkänen, Pyorala and Laakso (1995) | 1069 | ↑     | -     | ↑  |-
| Båvenholm, et al. (1995a)     | 103         | ↑     | ↑     | -  |-
| Båvenholm, et al. (1995b)     | 103         | ↑     | -     | -  |-
| Radhika, et al. (2009)        | 2042        | ↑     | -     | ↑  |-
| Mohan, et al. (2005)          | 100         | ↑     | -     | ↑  |-
| Snehalatha, et al. (2001)     | 82          | ↑     | ↑     | ↑  |-
| Ramachandran, et al. (1998a)  | 151         | ↑     | ↑     | ↑  |

Disability adjusted life years (DALYs), a commonly used metric of premature of death and disability, lost secondary to CAD in India have been predicted to raise from 7.67 million to 14.4 million in men and 5.6 million to 7.7 million in women from 2000 to 2020 (Gupta, et al., 2008). Despite the high prevalence of both CAD and metabolic risk factors for CAD in the South Asian Indian population, there are sparse and conflicting data linking the two in these populations (Pais, et al., 1996). The current study reports the relationship between insulin and other risk factors of CAD like, hypertension, blood glucose, and lipid levels in a case-control study carried out in South Indian population.

**Indian Population**

<table>
<thead>
<tr>
<th>Study</th>
<th>Sample Size</th>
<th>HDL-C</th>
<th>LDL-C</th>
<th>TG</th>
</tr>
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<tbody>
<tr>
<td>Radhika, et al. (2009)</td>
<td>2042</td>
<td>↑</td>
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<tr>
<td>Mohan, et al. (2005)</td>
<td>100</td>
<td>↑</td>
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<tr>
<td>Snehalatha, et al. (2001)</td>
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<tr>
<td>Ramachandran, et al. (1998a)</td>
<td>151</td>
<td>↑</td>
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</tr>
</tbody>
</table>
behind this overall confusing data may be due to the cross-reactivity between insulin and its pro-hormone, proinsulin, which was present in many assays for insulin estimation. Relatively few and conflicting data are available on the association of proinsulin to IR, CAD, and atherosclerosis (Bavenholm, et al., 1995b; Katz, et al., 1996; Yudkin, et al., 1997). Prospective studies have established that the proinsulin-to-insulin ratio determination may be utilized to predict worsening in glucose tolerance (Nijpels, et al., 1996; Haffner, et al., 1997). In later stages of type 2 diabetes, des 31, 32 proinsulin a commonly secreted by-product of Beta-cell secretion is considered to be involved in the development of macrovascular disease, whereas des 64, 65 is usually not seen in the circulation (Yudkin, 1993; Wareham, Byrne and Hales, 1995).

Cross-sectional relationships between proinsulin and other risk factors intermediate quantitative traits associated with CAD have been examined in a few numbers of studies especially in the Western population (Nagi, et al., 1990; Haffner, et al., 1993b; Mohamed-Ali, et al., 1995; Yudkin, et al., 1997; Grootenhuis, et al., 1998). Among both diabetic and non-diabetic subjects, proinsulin has demonstrated moderate but significant correlation with blood pressure (BP) and concentrations of total cholesterol, triglycerides, LDL-C and HDL-C (Nagi, et al., 1990; Haffner, et al., 1993a and 1993b; Mohamed-Ali, et al., 1995; Yudkin, et al., 1997; Grootenhuis, et al., 1998) and plasminogen activator inhibitor type 1 (Gray, et al., 1997) independent of other factors included in multivariate analysis. However, the role of elevated proinsulin as an independent player in atherogenesis remains debatable (Katz, et al., 1996; Yudkin, et al., 1997). There also exists some disagreement on the association of proinsulin with
cardiovascular risk factors in varied ethnic groups (Haffner, et al., 1993b; Yudkin, et al., 1997).

A chief research question is yet to be addressed in this body of literature. Are serum proinsulin concentrations associated with CAD risk factors among South Indians, a population with proven reports of higher prevalence of both type 2 diabetes and CAD? We examined these specific issues using data from the angiographically verified case control study.

2.3.3. Role of Proinsulin/Insulin ratio in CAD

Elevated proinsulin with proinsulin intermediates (collectively referred to as proinsulins) and proinsulin/insulin (P/I) ratios are hallmarks of type 2 diabetes with risk of CAD (Kim et al., 2000; Mako, Straff and Rubenstein, 1977; Ward, et al., 1987; Yoshioka, et al., 1988; Temple, et al., 1989) (see Table 1). The causes of these CAD abnormalities are insufficiently clarified. In particular, it is unresolved whether conditions intrinsic to the β-cell are an important cause of elevated proinsulins and P/I ratios. There exist mutations in the proinsulin molecule that inhibit the conversions of proinsulin to insulin, but these mutations are rare (Gabbay, et al., 1976). However, subtle alterations of the activities of proconvertase enzymes that convert proinsulin to insulin could potentially be widespread and have a genetic basis. A genetic basis is suggested from studies that found parallelisms between heredity for type 2 diabetes and elevated proinsulins and P/I ratios (Beer, et al., 1990; Haffner, et al., 1995; Ramachandran, et al., 1998b). Comparative studies between different populations show substantial differences in the P/I ratio (Nagi, et al., 1998), a finding also compatible with
genetic effects. However, other studies give evidence against a role for hereditary factors. Hence, a Danish twin study indicated no effect of heredity on proinsulins and P/I ratios (Roder, et al., 1995). Results of a Norwegian study also contest a role of heredity, since proinsulin abnormalities were not found in prediabetic subjects (Birkeland, et al., 1994).

A role of genetic factors for elevated proinsulins and P/I ratios is difficult to determine if the confounding influence of other factors is not accounted for. One such factor may be hyperglycemia. Many (Yoshioka, Kuzuya, Matsuda and Iwamoto, 1989; Davies, et al., 1994; Clauson, Alvarsson and Grill, 1997), but not all, clinical studies thus demonstrate that normalization of hyperglycemia in type 2 diabetes decreases proinsulins and P/I ratios. A positive relation has been demonstrated between the level of hyperglycemia and proinsulin in overt diabetes and CAD (Yoshioka, et al., 1989; Davies, et al., 1994; Clauson, Alvarsson and Grill, 1997). Other possibly confounding factors could be age, obesity, and birth weight. Age has been reported to be associated with proinsulin abnormalities (Shimizu, et al., 1996; Rachman, et al., 1997). Obesity with accompanying insulin resistance has been associated in some studies with hyperproinsulinemia (Wang, et al., 1997; Mykkanen, et al., 1997; Mykkänen et al. 1999) but not with elevated P/I ratios (Wang, et al., 1997; Mykkanen, et al., 1997; Mykkanen, et al., 1999; Shiraishi, et al., 1991). Low birth weight also gives rise to insulin resistance (Phillips, et al., 1994a) and may associate with proinsulin parameters (Stern, Bartley, Duggirala and Bradshaw, 2000).
In a prior study, data were assembled from a large, population-based study of middle-aged Swedish men who were tested for glucose tolerance by an oral glucose tolerance test (OGTT). The design of the study was to enrich the study population with subjects who had a strong family history of diabetes to provide one half of the study population. The results obtained from these subjects were then to be contrasted with the other, age-matched half of the study population consisting of subjects with no known relatives with diabetes. Results have been reported on the diabetogenic influence of family history of diabetes (Grill, et al., 1999), obesity (Carlsson, et al., 1998), and birth weight (Carlsson, et al., 1999) in this population. Although insulin resistance and decreased insulin secretion are characteristics of established type 2 DM, which of these metabolic abnormalities is the primary determinant of type 2 DM is controversial. It is also not well known how insulin resistance and beta cell dysfunction influence serum insulin, proinsulin, and proinsulin/insulin ratio in type 2 DM with CAD.

2.4. Materials and Methods

The levels of serum insulin and proinsulin were estimated in the study population, between the age group 35-65 years, admitted in the Cardiac Care Centre of Sri Ramachandra Hospital, affiliated to Sri Ramachandra University, Chennai. This investigation was carried out using a case control study. The subjects (n=125) with CAD confirmed by coronary angiogram were cases. The subjects (n=125) with absence of CAD confirmed by coronary angiogram comprised the control group.
2.4.1. Methods Adopted

Fasting venous blood samples (5ml) were collected from both case and control for SI and PI measurements. The serum thus separated was frozen until the assay for the estimations of SI and PI were performed. Serum concentrations of Insulin and Proinsulin were measured by solid phase enzyme linked immunosorbant assay techniques (ELISA) using UBI MAGIWEL Insulin kit (Mountainview, CA, USA) and DRG Proinsulin ELISA kits (DRG, International, Inc., USA) respectively. PI/SI ratio was determined in each patient (Herman, Hawthorne and Hamman, 1989). Insulin resistance was calculated using Homeostasis assessment (HOMA-IR) model using the formula: Fasting insulin (µIU/ml) x fasting glucose (mmol/litre) / 22.5, (Matthews, et al., 1985).

2.4.1.1. Estimation of Insulin using UBI MAGIWEL kit

Principle: UBI MAGIWEL Insulin kit is based on solid phase enzyme-linked immunosorbant assay (ELISA). The wells are coated with monoclonal antibody with higher activity for insulin. When the samples and controls are incubated in the wells with enzyme conjugate, which is another antibodies linked to horseradish peroxidase to form a sandwich complex bound to the well. Unbound conjugates are then washed off with wash buffer. The amount of bounded peroxidase is proportional to the concentration of the insulin present in the sample. Upon addition of the substrate and chromogen, the intensity of color will develop in proportional to the concentration of insulin in the samples.

Materials: 1. Microwell Strips (96 wells): Monoclonal Anti-Insulin Antibody coated wells.8x12 strips, 2. Enzyme Conjugate (11ml): Anti-Insulin Antibodies
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Procedure:

1. The desired number of coated wells in the holder were secured and date sheet with sample identification was marked
2. 25 uL of serum sample, controls and reference into the assigned wells were dispensed
3. 100 uL of enzyme conjugate was dispensed into each well and mixed for 5 seconds
4. The mixture was incubated for 30 minutes at 25°C
5. Incubation mixture was removed and the wells were rinsed five times with washing buffer
6. 100 uL of Solution A and then 100 uL of Solution B were dispensed into each well
7. The resultant mixture was incubated for 15 minutes at room temperature.
8. Reaction was stopped by adding 50 uL of 2N HCl to each well and O.D. was read at 450 nm with a microwell reader.
This assay is highly sensitive with the minimal detectable concentration of insulin is estimated to be 0.5 uU/mL. Also it is highly specific with essentially no cross reactivity with C-peptide at the concentration of 5000 pmol/mL, with intact human proinsulin (biosynthetic) 0.3%.

2.4.2.2. Estimation of Pro-insulin using DRG kit

The DRG Proinsulin ELISA is an enzyme immunoassay for the quantitative in vitro diagnostic measurement of Proinsulin (intact) in serum.

**Principle:** The DRG Proinsulin ELISA Kit is a solid phase enzyme-linked immunosorbent assay (ELISA) based on the sandwich principle. The microtiter wells are coated with a monoclonal antibody directed towards a unique antigenic site on a Proinsulin molecule. An aliquot of patient sample containing endogenous Proinsulin is incubated in the coated well with enzyme conjugate, which is an anti-proinsulin antibody conjugated with horseradish peroxidase. After incubation the unbound conjugate is washed off. The amount of bound peroxidase is proportional to the concentration of Proinsulin in the sample. Having added the substrate solution, the intensity of color developed is proportional to the concentration of Proinsulin in the patient sample.

**Reagents:**

1. Microtiterwells, 12x8 (break apart) strips, 96 wells; Wells coated with Anti-Proinsulin antibody (monoclonal).
2. Standard (Standard 0-5), 6 vials (lyophilized), 1 mL; Concentrations: 0 - 2.6 – 6.6 – 16.5 – 33 – 66 pmol/L Conversion: 106 pmol/L = 1 ng/mL. It contains < 0.0015% Proclin 300, ≤ 0.015% BND (5-bromo-5-nitro-1,3-
dioxane) and ≤ 0.010% MIT (2-methyl-2H-isothiazol-3-one) as preservative.

3. Control (low and high), 2 vials, (lyoph.), 2.0 mL (exact control ranges see vial label). It contains < 0.0015% Proclin 300, ≤ 0.015% BND and ≤ 0.010% MIT as preservative.

4. Sample Diluents, 1 vial, 2 mL, ready to use, it contains < 0.0015% Proclin 300, ≤ 0.015% BND and ≤ 0.010% MIT as preservative.

5. Enzyme Conjugate 11X concentrate, 1 vial, 1.2 mL, Anti-Proinsulin antibody conjugated to horseradish Peroxidase. It contains 0.015% BND and 0.010% MIT as preservative.

6. Conjugate Diluents, 1 vial, 12 mL, ready to use, it contains < 0.0015% Proclin 300, ≤ 0.015% BND and ≤ 0.010% MIT as preservative.

7. Assay Buffer, 1 vial, 12 mL, ready to use, it contains < 0.0015% Proclin 300, ≤ 0.015% BND and ≤ 0.010% MIT as preservative.

8. Substrate Solution, 1 vial, 14 mL, ready to use, Tetramethylbenzidine (TMB).

9. Stop Solution, 1 vial, 14 mL, ready to use, contains 0.5 M H₂SO₄

10. Wash Solution, 1 vial, 30 mL (40X concentrated),

**Procedure:**

**Each run must include a standard curve.**

1. The desired number of microtiter wells in the holder was secured.

2. 100 μL of each standard, control and samples with new disposable tips into appropriate wells was dispensed.

3. 100 μL assay buffer into each well was dispensed.
4. Mixed thoroughly for 10 seconds. It is important to have a complete mixing in this step.

5. The plate was covered with a plate sealer and incubated overnight (16-24 hours) at 4°C in a humidity chamber.

6. The contents of the wells were briskly shaken out. The wells were rinsed 3 times with diluted wash solution (350 μL per well). The wells were stroked sharply on absorbent paper to remove residual droplets.

7. 100 μL of diluted enzyme conjugate was dispensed into each well.

8. Mix for 10 seconds thoroughly. It is important to have a complete mixing in this step.

9. The resultant mixture was incubated for 60 minutes at room temperature (without covering the plate).

10. The contents of the wells were briskly shaken out. The wells were rinsed 5 times with diluted wash solution (350 μL per well). The wells were stroked on absorbent paper to remove residual droplets.

11. 100 μL of substrate solution was added to each well.

12. Later incubated for 30 minutes at room temperature.

13. The enzymatic reaction was stopped by adding 50 μL of stop solution to each well.

14. The OD at 450±10 nm was read with a microtiter plate reader within 10 minutes after adding the stop solution.

2.4.2. Statistical Analysis

Descriptive statistics were used to summarize the clinical findings, risk factors for CAD. Student’s t test and Chi square analysis were used to get the
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statistical significance. Pearson’s correlation analysis was used to find the associations of insulin, proinsulin and proinsulin/insulin ratio with other cardiovascular risk factors. The multiple logistic regression analysis was used to estimate the effect of insulin and proinsulin on CAD, controlling the other confounders. As the distribution of insulin and proinsulin were highly skewed, logarithmic transformation of Insulin and proinsulin were used for statistical analysis. A value of P<0.05 was taken as significant.

2.5. Results

In the current study among 125 subjects diagnosed with CAD, 91 (73%) patients were males and 34 (27%) were females. In angiographically proven control population 71 (57%) were females and 54 (43%) were males. Among cases 34 (27.2%), 35 (28%) and 56 (44.8%) had SVD, DVD and TVD respectively. 73 (58.4%) subjects had type 2 DM with CAD and 52 (41.6%) had CAD without type 2 DM. Among the control subjects, 64 (51.2%) subjects had type 2 DM and 61 (48.8%) had no type 2 DM. In the total study population, 111 (44.4%) subjects had positive family history of CAD while 139 (55.6%) subjects had no family history of CAD. Among the above study population 198 (79.2%) were non smokers. 37 (14.8%) were smokers and 15 (6%) were ex-smokers.

Table-2 shows characteristics of subjects with CAD and without CAD included in the case control study such as, age, body mass index (weight in kg / height in m²), waist hip ratio and waist circumference, systolic blood pressure (mm Hg), diastolic blood pressure (mm Hg), glycated hemoglobin (%), total serum cholesterol (mg/dl), serum triglycerides (mg/dl), total serum cholesterol (mg/dl),...
Insulin, Proinsulin and CAD

serum triglycerides (mg/dl), LDL-C (mg/dl), HDL-C (mg/dl). We found that subjects with CAD when compared without CAD had higher systolic blood pressure (139 vs. 128 mm Hg, P < 0.01), diastolic pressure (87 vs. 82 mm Hg, P < 0.01), fasting plasma glucose (144 vs. 127 mg/dl, P < 0.05) and glycated haemoglobin (7.0 vs. 6.5 %, P < 0.05). However, waist hip ratio (WHR) (94.4 vs. 93.2 cm, P < 0.368), LDL-C (109 vs. 107 mg/dl, P= 0.550) and HDL-C (40 vs. 41 mg/dl, P= 0.213) levels were not statistically significant between the subjects with CAD and without CAD.

Table 2: General characteristics of study subjects

<table>
<thead>
<tr>
<th>Variables*</th>
<th>Subjects without CAD (n = 125)</th>
<th>Subjects with CAD (n = 125)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>51 ± 8</td>
<td>55 ± 8</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>26.5 ± 4.3</td>
<td>26.2 ± 4.0</td>
<td>0.333</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>93.2 ± 11.4</td>
<td>94.4 ± 11.1</td>
<td>0.368</td>
</tr>
<tr>
<td>Systolic blood pressure (mm Hg)</td>
<td>128 ± 16</td>
<td>139 ± 19</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Diastolic blood pressure (mm Hg)</td>
<td>82 ± 9</td>
<td>87 ± 9</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>HOMA – IR</td>
<td>3.6 ± 1.5</td>
<td>5.4 ± 1.7</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Fasting plasma glucose (mg/dl)</td>
<td>127 ± 51</td>
<td>144 ± 66</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Glycated haemoglobin (%)</td>
<td>6.5 ± 1.4</td>
<td>7.0 ± 1.6</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Total serum cholesterol (mg/dl)</td>
<td>166 ± 42</td>
<td>172 ± 39</td>
<td>0.187</td>
</tr>
<tr>
<td>Serum triglycerides (mg/dl)</td>
<td>159 ± 86</td>
<td>185 ± 83</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>LDL cholesterol (mg/dl)</td>
<td>107 ± 30</td>
<td>109 ± 30</td>
<td>0.550</td>
</tr>
<tr>
<td>HDL cholesterol (mg/dl)</td>
<td>41 ± 7</td>
<td>40 ± 7</td>
<td>0.213</td>
</tr>
<tr>
<td>hs-CRP (mg/dl)</td>
<td>0.44 ± 0.12</td>
<td>0.55 ± 0.13</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

* Data is presented as mean± SD
The relationships of CAD risk factors with insulin, pro-insulin and proinsulin/insulin ratio among the study population are shown in Table 3. Fasting serum insulin level was associated directly with FBS, HbA1C and HOMA IR (P < 0.001). In addition, statistically significant association was observed between serum insulin and the following biochemical parameters like Lp(a), TG/HDL-C ratio (P < 0.01) and hs-CRP levels (P < 0.05). The elevated level of BMI (r=0.136, P=0.032) was significantly correlated with proinsulin concentration. Also, the higher level of BMI (r=0.192, P<0.002), and waist circumference (r=0.140, P=0.028) values were very highly significant with proinsulin/insulin ratio which was responsible for CAD among patients. Serum insulin levels were found to be significantly associated with all the cardiac markers (P < 0.001). Also the proinsulin/insulin ratio had significant association with cardiac markers like CPK (P < 0.001), CPKMB and LDH (P < 0.05). In addition serum insulin level significantly correlated with the stenosis score (P< 0.05).

The subjects with CAD had higher concentrations of SI (Mean ± SEM (standard error of the mean) = 13.79±0.76; P=0.013), PI (Mean ± SEM = 2.77±0.20; P=0.009) and lower PI/SI ratio (Mean ± SEM = 0.29±0.37; P=0.021) when compared with those subjects without CAD, i.e. SI (Mean ± SEM = 10.79±0.97), PI (Mean ± SEM = 2.07±0.16) and PI/SI ratio (Mean ± SEM = 0.48±0.69) respectively. The levels of SI, PI and PI/SI ratio were significantly associated with CAD (P<0.05) (Figure 8, 9 and 10).
Table 3: Pearson correlation analysis of CAD risk factors for Insulin, Proinsulin and Proinsulin-Insulin ratio

<table>
<thead>
<tr>
<th>Risk Factors</th>
<th>Insulin (uIU/L)</th>
<th>Proinsulin (uIU/L)</th>
<th>Proinsulin – Insulin Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>r value</td>
<td>P value</td>
<td>r value</td>
</tr>
<tr>
<td>Age (Years)</td>
<td>0.016</td>
<td>0.802</td>
<td>0.008</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>0.073</td>
<td>0.253</td>
<td><strong>0.136</strong></td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>0.007</td>
<td>0.910</td>
<td>0.113</td>
</tr>
<tr>
<td>Waist Hip Ratio</td>
<td>0.011</td>
<td>0.858</td>
<td>0.119</td>
</tr>
<tr>
<td>Systolic BP (mm Hg)</td>
<td>0.074</td>
<td>0.243</td>
<td>0.031</td>
</tr>
<tr>
<td>Diastolic BP (mm Hg)</td>
<td>0.084</td>
<td>0.186</td>
<td>0.024</td>
</tr>
<tr>
<td>Fasting blood sugar (mg/dl)</td>
<td><strong>0.344</strong></td>
<td>&lt; 0.001</td>
<td>0.018</td>
</tr>
<tr>
<td>Glycated hemoglobin (%)</td>
<td><strong>0.420</strong></td>
<td>&lt; 0.001</td>
<td>0.015</td>
</tr>
<tr>
<td>HOMA IR</td>
<td><strong>0.759</strong></td>
<td>&lt; 0.001</td>
<td>-0.016</td>
</tr>
<tr>
<td>Serum cholesterol (mg/dl)</td>
<td>0.058</td>
<td>0.358</td>
<td>0.013</td>
</tr>
<tr>
<td>HDL cholesterol (mg/dl)</td>
<td>0.023</td>
<td>0.721</td>
<td>0.060</td>
</tr>
<tr>
<td>LDL cholesterol (mg/dl)</td>
<td>0.037</td>
<td>0.561</td>
<td>0.027</td>
</tr>
<tr>
<td>Non HDL cholesterol (mg/dl)</td>
<td>0.056</td>
<td>0.411</td>
<td>0.042</td>
</tr>
<tr>
<td>TG (mg/dl)</td>
<td>0.084</td>
<td>0.190</td>
<td>0.039</td>
</tr>
<tr>
<td>TG : HDL</td>
<td><strong>0.269</strong></td>
<td>&lt; 0.001</td>
<td>0.028</td>
</tr>
<tr>
<td>TC: HDL</td>
<td>0.032</td>
<td>0.6714</td>
<td>0.080</td>
</tr>
<tr>
<td>Lp(a) (mg/dl)</td>
<td><strong>0.171</strong></td>
<td>0.007</td>
<td>-0.036</td>
</tr>
<tr>
<td>hs-CRP (mg/dl)</td>
<td><strong>0.156</strong></td>
<td>0.014</td>
<td>0.076</td>
</tr>
<tr>
<td>Cardiac Markers</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CPK (u/l)</td>
<td><strong>0.253</strong></td>
<td>&lt; 0.001</td>
<td>0.060</td>
</tr>
<tr>
<td>CPK-MB (u/l)</td>
<td><strong>0.241</strong></td>
<td>&lt; 0.001</td>
<td>0.040</td>
</tr>
<tr>
<td>LDH (u/l)</td>
<td><strong>0.258</strong></td>
<td>&lt; 0.001</td>
<td>0.102</td>
</tr>
<tr>
<td>Troponin t (ng/dl)</td>
<td><strong>0.185</strong></td>
<td>&lt; 0.001</td>
<td>0.016</td>
</tr>
<tr>
<td>Stenosis score</td>
<td><strong>0.163</strong></td>
<td>0.010</td>
<td>0.070</td>
</tr>
</tbody>
</table>

** - Correlation is highly significant at the 0.01 level (2-tailed)
* - Correlation is significant at the 0.05 level (2-tailed)
Insulin, Proinsulin and CAD

Figure 8: Serum Insulin levels in relation to CAD

Figure 9: Serum Proinsulin levels in relation to CAD

Figure 10: Serum Proinsulin-insulin ratio levels in relation to CAD
An investigation into the relationship of insulin and other related biochemical parameters with coronary artery disease in a South Indian population

**Figure 11: Serum Insulin levels in relation to CAD and DM**

**Figure 12: Serum Proinsulin levels in relation to CAD and DM**

**Figure 13: Serum Proinsulin-Insulin ratio in relation to CAD and DM**
Further, the mean insulin level in CAD subjects with DM (Mean ± SEM=13.81±1.27; was higher when compared with non-CAD subjects without DM (Mean ± SEM=10.50±1.33; P<0.05), (Figure 11). The mean proinsulin level was higher in CAD subjects with DM (Mean ± SEM=3.29±0.38) compared to non-CAD subjects without DM (Mean ± SEM=1.83±0.231; P<0.05) which was highly significant (Figure 12). Finally, the mean level of proinsulin/insulin ratio in subjects with CAD and DM (Mean ± SEM= 0.26±0.050) was lower when compared to non-CAD subjects with DM (Mean ± SEM= 0.62±0.12; P<0.05), (Figure 13) which was highly significant. The above findings indicated that in subjects diagnosed with CAD and DM, insulin and proinsulin mean levels were responsible for the high prevalence of CAD among this population.

Multiple logistic regression analysis was carried out keeping insulin and proinsulin as independent variables and CAD as a dependent variable (Table 4). Subsequently the models were Model 1: SI and PI unadjusted, Model 2: model 1 + adjusted for age, Model 3: model 2 + adjusted for gender, Model 4: model 3 + adjusted for waist circumference for the independent variables for insulin and proinsulin. In this case, the models of insulin had significant and positive association with CAD. The trend for proinsulin was as follows: Model 1 - (OR: 1.175; p < 0.01; 95% CI: 1.037 – 1.332), Model 2 - (OR: 1.168; p < 0.01; 95% CI: 1.028 – 1.327), Model 3 - (OR: 1.120; p=0.094; 95% CI: 0.981 – 1.280), Model 4 - (OR: 1.121; p=0.093; 95% CI: 0.981 – 1.281). While for insulin the trend was as follows: Model 1 - (OR: 1.030; p < 0.01; 95% CI: 1.005 – 1.055), Model 2 - (OR: 1.031; p < 0.01; 95% CI: 1.006 – 1.057), Model 3 - (OR: 1.035; p < 0.01; 95% CI
1.008 – 1.062), Model 4 - (OR: 1.035; p < 0.01; 95% CI: 1.008 – 1.062); Since serum insulin remained statistically significant, logistic regression analysis was further extended by adjusting for the other confounders hypertension (Model 5) and serum cholesterol (Model 6). The trend was as follows: Model 5 - (OR: 1.031; p < 0.05; 95% CI: 1.005 – 1.059); Model 6 - (OR: 1.029; p < 0.05; 95% CI: 1.002 – 1.056). Thus serum insulin proved to be a better independent risk factor for CAD, even after adjustment with the confounding variables like age, gender, waist circumference, hypertension and total cholesterol, whereas proinsulin lost its significance when adjusted for the confounders gender and waist circumference.

Table 4: Multiple logistic regression analysis using CAD as dependent variable

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Odds Ratio [OR]</th>
<th>95% Confidence Interval [CI]</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Independent variable: Insulin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Model 1: Insulin – Unadjusted</td>
<td>1.030</td>
<td>1.005 – 1.055</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Model 2: [Model 1 + adjusted for age]</td>
<td>1.031</td>
<td>1.006 – 1.057</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Model 3: [Model 2 + adjusted for gender]</td>
<td>1.035</td>
<td>1.008 – 1.062</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Model 4: [Model 3 + adjusted for waist circumference]</td>
<td>1.035</td>
<td>1.008 – 1.062</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Model 5: [Model 4 + adjusted for hypertension]</td>
<td>1.031</td>
<td>1.005-1.059</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Model 6: [Model 5 + adjusted for total cholesterol]</td>
<td>1.029</td>
<td>1.002-1.056</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Independent variable: Proinsulin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Model 1: Proinsulin – Unadjusted</td>
<td>1.175</td>
<td>1.037 – 1.332</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Model 2: [Model 1 + adjusted for age]</td>
<td>1.168</td>
<td>1.028 – 1.327</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Model 3: [Model 2 + adjusted for gender]</td>
<td>1.120</td>
<td>0.981 – 1.280</td>
<td>0.094</td>
</tr>
<tr>
<td>Model 4: [Model 3 + adjusted for waist circumference]</td>
<td>1.121</td>
<td>0.981 – 1.281</td>
<td>0.093</td>
</tr>
</tbody>
</table>
### Table 5: Chi-Square Tests for association of CAD with Insulin and Proinsulin

<table>
<thead>
<tr>
<th></th>
<th>Insulin</th>
<th></th>
<th>Proinsulin</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Value</td>
<td>Asymp. Sig. 2-sided</td>
<td>Exact Sig. 2-sided</td>
<td>Exact Sig. 1-sided</td>
</tr>
<tr>
<td>Pearson Chi-Square</td>
<td>13.269&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.000</td>
<td>3.136&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.077</td>
</tr>
<tr>
<td>Continuity Correction&lt;sup&gt;b&lt;/sup&gt;</td>
<td>12.354</td>
<td>0.000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Likelihood Ratio</td>
<td>13.392</td>
<td>0.000</td>
<td>3.143</td>
<td>0.076</td>
</tr>
<tr>
<td>Fisher’s Exact test</td>
<td></td>
<td></td>
<td>13.215</td>
<td>0.000</td>
</tr>
<tr>
<td>Linear-by-Linear Association</td>
<td></td>
<td></td>
<td>13.124</td>
<td>0.077</td>
</tr>
<tr>
<td>N of Valid Cases</td>
<td>250</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

#### Notes:
- a. 0 cells (0.0%) have expected count less than 5. The minimum expected count is 60.25
- b. Computed only for a 2x2 table
- c. 0 cells (0.0%) have expected count less than 5. The minimum expected count is 62.00
- d. Computed only for a 2x2 table

The Chi-Square tests were performed to measure the value (presence or absence of normal and abnormal) of CAD risk factors such as insulin and proinsulin, among the valid subjects (Table 5). The 2-tailed test revealed, that the values of serum Insulin (13.269; P<0.000) and that of proinsulin (3.136; P<0.077) among valid subjects respectively for prediction of CAD. In this case, proinsulin had shown very lower Chi-Square value, continuity correction, likelihood ratio and linear- by- linear association in 2-tailed test than insulin. Also, the Fischer’s exact value for proinsulin in exact-2-tailed and exact-1-tailed test had shown less...
significance for prediction of CAD than Insulin. Hence, insulin is comparatively a better prospective predictor of CAD than proinsulin.

2.6. Discussion

This case control study clearly shows that high serum insulin and proinsulin (PI) levels are independently associated with the risk of diabetic and non-diabetic angiographically documented CAD subjects in South Indians. These results are consistent with data from other studies suggesting a progressive relationship between serum insulin levels and an increased risk of CAD in both non-diabetic (Scheidt-Nave, et al., 1991; Moss, et al., 1994; Park, et al., 1996) and diabetic patients (McKeigue, et al., 1993; Moss, Klein, R., Klein, B. E. and Meuer, 1994; Kuusisto, Mykkanen, Pyorala and Laakso, 1994b; Andersson and Svardsudd, 1995).

This cross-sectional case control study showed that hyperinsulinaemia and hyperproinsulinaemia are associated with CAD, even in non-diabetic subjects. Several studies have shown that IR is a characteristic feature in Asian Indians (Ahuja, 1996; Yudkin, et al., 1997; Ramachandran, et al., 1998a; Bhopal, et al., 1999; Snehalatha, et al., 2001; Mohan, et al., 2005; Radhika, et al., 2009). Components of syndrome ‘X’, namely obesity, upper body adiposity, IR and dyslipidaemia, representing a very complex metabolic and cardiovascular web, occurs even in the non-existence of hyperglycaemia. With the progressing diabetes and subsequent worsening of insulin metabolism, the anomalies related to hyperinsulinaemia, IR, and hyperproinsulinaemia are likely to exacerbate.
An increased PI to SI ratio has been reported in Type 2 diabetes in several cross-sectional studies (Yoshioka, et al., 1988; Sadd, et al., 1990; Ramachandran, et al., 1998). Yudkin, et al., (1997) assessed the potential risk of Proinsulin for CAD in non-diabetic European and South Asian subjects in a prospective study. The results indicated that PI-like molecules were predictors of CAD independently of insulin, but unlikely to be involved directly in the etiopathogenesis of CAD, in both races. But our study showed a strong independent association of Serum Insulin and Proinsulin in diabetic and non diabetic CAD subjects in the South Indian population. The predictive nature of the finding can be evaluated only by a prospective study. Haffner, et al., (1993b), in the San Antonio heart study, found PI to be strongly predictive of several metabolic and haemodynamic variables in non-diabetic subjects. The presence of hyperproinsulinaemia reflects abnormal processing of the PI molecule. The association of a high concentration of serum insulin and proinsulin-like molecules with CAD risk factors in Type 2 diabetes has been demonstrated in some prospective studies (Welborn and Wearne, 1979; Yudkin, et al., 1997), and cross sectional studies on global population (Bavenholm, et al., 1995a; Bavenholm, et al., 1995b; Kahn, et al., 1995; Kuusisto, Mykkanen, Pyorala and Laakso, 1995; Haffner, et al., 1998b; Lempiainen, et al., 1999; Gerstein, et al., 1999; Matsumoto, et al., 1999; Kim, et al., 2000; Sheu, et al., 2000; Lakka, et al., 2000; Matsumoto, et al., 2001; Kuusisto, Lempiainen, Mykkanen and Laakso, 2001; Ferrara and Goldberg, et al., 2001; Hanley, et al., 2001; Oh, Barrett-Connor and Wedick, 2002; Yudkin, et al., 2002; Amoah, et al., 2002; Zethelius, et al., 2002; Dabelea, et al., 2003; Pfutzner, et al., 2004; Drexel, et al., 2005; Alssema, et al., 2005; Quadros, et al., 2006; Gui, et al., 2008; Tanaka,
et al., 2010; Acibucu, Kayatas and Candan et al., 2010; Tanaka, et al., 2010; Heck, et al., 2010; Schauer, et al., 2011; the current study corroborates with the findings of the afore mentioned studies.

To our knowledge, there have been five previous western-studies exploring an association between insulin level and/or insulin sensitivity and angiographically documented CAD (Shinozaki, et al., 1996; Bressler, et al., 1996; Takezako, et al., 1999; Min, 1996). Most studies have shown that patients with CAD were hyperinsulinemic when compared with those subjects without CAD only in normal glucose tolerance group. However the studies regarding serum insulin and proinsulin levels with angiographically documented CAD with and without diabetes are lacking from South India.

In our study multiple regression analysis revealed that the serum insulin associated risk persisted even after controlling for other risk factors suggesting that serum insulin is an independent and better marker for CAD than its precursor - proinsulin, in South Indians. This finding corroborated with the findings of Kahn, et al., (1995). Many other studies have given mixed and conflicting reports.

Laakso, et al., (1991) demonstrated that insulin resistance but not hyperinsulinemia was related to asymptomatic atherosclerosis in the femoral or carotid arteries. Certain studies demonstrated that, Proinsulin is increased relative to insulin in subjects with type 2 diabetes (Ward, et al., 1987; Yoshioka, et al., 1988; Temple, et al., 1989); elevated in subjects with impaired glucose tolerance (Davies, et al., 1993; Haffner, et al., 1994b), but not in all (Saad, et al., 1990) studies. In some studies elevated proinsulin is more strongly correlated with
Insulin, Proinsulin and CAD


However, Kahn and colleagues (Kahn, et al., 1995) reported no association between proinsulin and CAD independent of diabetes status in Japanese-American men. In contrast, Bavenholm, et al., (1995a) found a significant relation between both insulin and proinsulin with CAD in 62 men; however these associations were no longer significant after adjustment for the confounder body mass index (BMI). While Yudkin, et al, (1997) found modest (but significant) relations between both insulin and proinsulin and prevalent CAD; but these results were no longer significant after adjustment for BMI. In few earlier studies of proinsulin with atherosclerosis, no significant associations were observed (Niskanen, et al., 1996: Katz, et al., 1996). Previously, Haffner and colleagues (Haffner, et al., 1998b) reported that the association between proinsulin and carotid intima-media wall thickness, a marker for atherosclerosis, was stronger than insulin by correlation analyses. Meantime, increased carotid artery intima-media thickness has been associated with both prevalent CAD (Burke, et al., 1995) and diabetes (Folsom, et al., 1994).

Some more studies have shown that absolute concentrations of proinsulin are higher together with insulin, in obese and insulin-resistant subjects (Nagi, et al., 1998; Saad, et al., 1990; Phillips, et al., 1994b; Anthony, Godsland and Stevenson, 1994; Mykkanen, et al., 1999; Wang, et al., 1997; Mykkanen, et al., 1997). More contentious whether proinsulin to insulin ratios are higher in insulin-resistant
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subjects, as well as being associated with reduced beta-cell function (Roder, et al., 1994; Phillips, et al., 1994b; Mykkänen, et al., 1997 and 1999). Studies have related relative proinsulin concentrations to measure insulin sensitivity and suggested that insulin-resistant subjects have normal or lower proinsulin to insulin ratios (Phillips, et al., 1994b; Wang, et al., 1997; Mykkänen, et al., 1997) and other studies have no increase in these ratios in obesity (Anthony, et al., 1994) or in highly insulin resistant cohorts (Nagi, et al., 1998). Our results are correlated with some cross-sectional studies (Welborn and Wearne, 1979; Yudkin, et al., 1997) and showing elevated levels of SI (Davies, et al., 1993; Haffner, et al., 1994a: Shinozaki, et al., 1996; Min, 1996; Bressler, et al., 1996; Takezako, et al., 1999; Seibaek, et al., 1997), PI (Ward, et al., 1987; Yoshioka, et al., 1988; Temple, et al., 1989; Nagi, et al., 1990; Laakso, et al., 1991; Haffner, 1993b; Haffner, et al., 1994b; Bavenholm, et al., 1995; Lindahl, et al., 1999; Yudkin, et al., 1997), and lower level of PI/SI ratio (Niskanen, et al., 1996: Katz, et al., 1996), those previous studies are all akin to our results. Our findings have revealed that, hyperinsulinemia per se may have a direct relation to coronary atherosclerosis in South Indian type 2 diabetic patients with CAD.

Moreover several prospective studies including a large number of type 2 diabetic patients have shown that glycemic control is an important factor for cardiovascular risk (Kuusisto, Mykkanen, Pyorala and Laakso, 1994b; Andersson and Svardsudd, 1995; Coutinho, et al., 1999). Several clinical observations showed that post-challenge hyperglycemia rather than fasting glucose was a strong risk factor of CAD (Barrett-Connor and Ferrara, 1998; Shaw, et al., 1999; Tominaga, et al., 1999; Saydah, et al., 2001). Temelkova-Kurktschiev, et al., (2000) reported that
2-h glucose was a significant independent determinant of intima-media thickness, although there were no coronary angiographic data. These reports are in agreement with our results that glucose intolerance as revealed by hyperinsulinemia was independently associated with the presence of coronary atherosclerosis.

Additionally, low-grade inflammation and a possible inherent genetic susceptibility are other contributing factors for CAD. In the present study serum insulin levels significantly correlated with cardiac markers and stenosis scores. Also serum insulin levels had significant associations with serum Lp(a) and hs-CRP levels. Increased levels of Lp(a) potentially delays thrombolysis and contribute to plaque progression. Thus insulin contributes synergistically with other risk factors in the pathophysiology of CAD. Further in multiple logistic regression analysis, we found that serum insulin was independently associated with CAD and proved to be a better risk factor than proinsulin when adjusted for confounders like age, gender, waist circumference and a variety of atherosclerotic risk factors. Also the chi square analysis proved the higher predictability of Insulin for CAD risk.

These findings suggest one of two possibilities: (1) that insulin and proinsulin-like molecules have actions, either through insulin receptor and proinsulin receptor (Jehle, Lutz and Fussgaenger, 1996) or through other biological mechanisms, that play some role in atherothrombosis; or (2) that higher concentrations of Serum-insulin and proinsulin-like molecules reflect another unmeasured common antecedent such as concentrations of proinflammatory cytokines (Yudkin, et al., 1999).
2.7. Conclusion

The current study investigated the relationship between serum insulin, proinsulin and proinsulin/insulin ratio and several biochemical markers which represent insulin resistance or beta cell function, in patients with CAD and controls with and without DM. Insulin and proinsulin were significantly higher in CAD patients with type 2 DM than control. The association between elevated levels of Insulin in CAD risk appears to be stronger than proinsulin. The current study proposes that hyperinsulinaemia has a vital and etiological role as a risk factor for risk factors in the sequence of events progressing towards increased risk of CAD.

The finding of a graded risk of CAD with glucose elevations within the “normal and abnormal” range in South Indians strongly supports the need to explore this relationship in other ethnic groups. If these observations are confirmed, the population attributable risk of CAD may be several times greater than the population attributable risk of diabetes alone. This would focus attention on the high prevalence of elevated glucose levels in the non-diabetic population and may lead to innovative ways of preventing CAD in this group.

In summary, IR, elevated levels of insulin, and proinsulin were found to be associated with CAD in glycaemic and normoglycaemic South Indian population. Serum insulin was a better predictor when compared to proinsulin for CAD risk. The findings of this study may have great relevance in this population which is currently facing a double epidemic burden of diabetes and CAD.
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